

- 1 -

ALANINE 2,3-AMINOMUTASE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Patent Applications 60/350,727 filed January 18, 5 2002 and 60/375,785 filed April 25, 2002.

FIELD

This disclosure relates to alanine 2,3-aminomutase nucleic acid and amino acid sequences, 10 cells having alanine 2,3-aminomutase activity which can convert alpha-alanine to beta-alanine, and methods using these cells to make beta-alanine, pantothenic acid, 3-hydroxypropionic acid, and other organic compounds.

BACKGROUND

Organic chemicals such as organic acids, esters, and polyols can be used to synthesize 15 plastic materials and other products. To meet the increasing demand for organic chemicals, more efficient and cost-effective production methods are being developed which utilize raw materials based on carbohydrates rather than hydrocarbons. For example, certain bacteria have been used to produce large quantities of lactic acid used in the production of polylactic acid.

3-hydroxypropionic acid (3-HP) is an organic acid. Several chemical synthesis routes have 20 been described to produce 3-HP, and biocatalytic routes have also been disclosed (WO 01/16346 to Suthers *et al.*). 3-HP has utility for specialty synthesis and can be converted to commercially important intermediates by known art in the chemical industry, e.g., acrylic acid by dehydration, malonic acid by oxidation, esters by esterification reactions with alcohols, and 1,3-propanediol by reduction.

25

SUMMARY

The compound 3-hydroxypropionic acid (3-HP) can be produced biocatalytically from PEP or pyruvate, through a key beta-alanine intermediate (FIG. 1). Beta-alanine can be synthesized in cells from carnosine, beta-alanyl arginine, beta-alanyl lysine, uracil via 5,6-dihydrouracil and N- 30 carbamoyl-beta-alanine, N-acetyl-beta-alanine, anserine, or aspartate (FIGS. 1 and 2). However, these routes are relatively inefficient because they require rare precursors or starting compounds that are more valuable than 3-HP.

Therefore, production of 3-HP using biocatalytic routes would be more efficient if alpha-alanine could be converted to beta-alanine directly (FIG. 1). Unfortunately, an enzyme that 35 interconverts alpha-alanine to beta-alanine has not yet been identified. It would be advantageous if enzymatic activities that carry out the conversion of alpha-alanine to beta-alanine were identified, such as an alanine 2,3-aminomutase.

Herein disclosed are alanine 2,3-aminomutase nucleic acid sequences (such as SEQ ID NOS: 20 and 29), amino acid sequences (such as SEQ ID NOS: 21 and 30), as well as variants, fragments, fusions, and polymorphisms thereof that retain alanine 2,3-aminomutase activity. In one example, the polypeptide is a sequence that includes SEQ ID NO: 21 or 30, or variants, fragments, or fusions thereof that retain alanine 2,3-aminomutase activity. In one example, the polypeptide is a mutated lysine 2,3-aminomutase and/or a lysine 5,6-aminomutase amino acid sequence. The disclosed sequences can be used to transform cells, such that the transformed cells have alanine 2,3-aminomutase activity, which allows the cells to produce beta-alanine from alpha-alanine. Binding agents that specifically bind to an alanine 2,3-aminomutase are encompassed by this disclosure.

Cells having alanine 2,3-aminomutase activity, which allow the cell to convert alpha-alanine to beta-alanine, are disclosed. Such cells can be eukaryotic or prokaryotic cells, such as yeast cells, plant cells, *Lactobacillus*, *Lactococcus*, *Bacillus*, or *Escherichia* cells. In one example, the cell is transformed with a mutated lysine 2,3-aminomutase and/or a mutated lysine 5,6-aminomutase that confers to the transformed cells alanine 2,3-aminomutase activity. In another example, transformed cells include an alanine 2,3-aminomutase, such as SEQ ID NO: 21 or 30. The disclosed cells can be used to produce nucleic acid molecules, polypeptides, and organic compounds. The polypeptides can be used to catalyze the formation of organic compounds or can be used as antigens to create specific binding agents.

A production cell having at least one exogenous nucleic acid, such as a nucleic acid encoding for an alanine 2,3-aminomutase, is disclosed. In one example, the nucleic acid sequence includes SEQ ID NOS: 20 or 29 (or fragments, variants, or fusions thereof that retain alanine 2,3-aminomutase activity). In another example, the nucleic acid sequence encodes an amino acid sequence shown in SEQ ID NO: 21 or 30 (or fragments, variants or fusion proteins that of that retain alanine 2,3-aminomutase activity). Production cells can be used to express polypeptides that have an enzymatic activity such as CoA transferase activity, beta-alanine ammonia lyase activity, 3-hydroxypropionyl-CoA (3-HP-CoA) dehydratase activity, glutamate dehydrogenase, 3-hydroxypropionyl-CoA hydrolase, alanine dehydrogenase, pyruvate-glutamate transaminase, and/or 3-hydroxyisobutyryl-CoA hydrolase activity. In another example, production cells are used to express polypeptides that have an enzymatic activity such as beta-alanine – 2-oxoglutarate aminotransferase and 3-HP dehydrogenase and/or 3-hydroxyisobutyrate dehydrogenase. Methods of producing polypeptides encoded by the nucleic acid sequences described above are disclosed.

A method of identifying a cell having alanine 2,3-aminomutase activity is disclosed. The method includes culturing a cell, which is functionally deleted for *panD*, in media which does not include beta-alanine nor pantothenate. For example, the cell can produce alpha-alanine from media sources of carbon, oxygen, hydrogen, and nitrogen, but which does not include beta-alanine. Cells capable of growing in the media are identified, wherein cell growth indicates that the cell is producing beta-alanine from alpha-alanine, which indicates the cell has alanine 2,3-aminomutase activity. In contrast, absence of cell growth indicates that the cell is not producing beta-alanine from

alpha-alanine, which indicates the cell does not have alanine 2,3-aminomutase activity. In one example, prior to culturing the cell for selection, cells are transformed with one or more mutated lysine 2,3-aminomutases and/or lysine 5,6-aminomutases.

A method of producing a polypeptide having alanine 2,3-aminomutase activity is disclosed.

5 In one example, the method includes culturing cells having at least one exogenous nucleic acid molecule that encodes an alanine 2,3-aminomutase (such as SEQ ID NOS: 20 and 29), which is capable of producing beta-alanine from alpha-alanine.

10 Several methods of producing 3-HP from beta-alanine using the disclosed cells having alanine 2,3-aminomutase activity are disclosed. In one example, the cell is transfected with one or more enzymes necessary to convert 3-HP from beta-alanine. In another example, the method includes purifying beta-alanine from the cell, then contacting the beta-alanine with polypeptides necessary to convert 3-HP from beta-alanine.

15 The cells, alanine 2,3-aminomutase nucleic and amino acid sequences (such as SEQ ID NOS: 20, 21, 29, and 30), and methods disclosed herein, can be used to produce pantothenate, 3-HP, and derivatives thereof such as coenzyme A (CoA), and other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, co-polymers of 3-HP and other compounds such as butyrates, valerates and other compounds, esters of 3-HP, and malonic acid and its esters. 3-HP is both biologically and commercially important. For example, the nutritional industry can use 3-HP as a food, feed additive or preservative, while the derivatives mentioned above can be produced from 3-HP.

20 Nucleic acid molecules encoding for an alanine 2,3-aminomutase (such as SEQ ID NOS: 20 and 29) can be used to engineer host cells with the ability to produce 3-HP as well as other organic compounds such as those listed above. Alanine 2,3-aminomutase peptides (such as SEQ ID NOS: 21 and 30) can be used in cell-free systems to make 3-HP as well as other organic compounds such as those listed above. The cells described herein can be used in culture systems to produce large quantities of 3-HP as well as other organic compounds such as those listed above.

25 One aspect of the disclosure provides cells, which in addition to alanine 2,3-aminomutase activity, include other enzyme activities, such as CoA transferase activity, beta-alanyl-CoA ammonia lyase activity, and 3-hydroxypropionyl-CoA dehydratase activity. In addition, methods of making products from these cells are disclosed. In some examples, the cell also includes one or more exogenous nucleic acid molecules that encodes one or more polypeptides having: glutamate dehydrogenase activity, CoA transferase activity, 3-hydroxypropionyl-CoA hydrolase, and/or 3-hydroxyisobutyryl-CoA hydrolase activity, and alanine dehydrogenase or pyruvate-glutamate transaminase activity. In another example, the cell also includes 4-aminobutyrate and/or beta-alanine-2-oxoglutarate aminotransferase activity and 3-HP dehydrogenase activity and/or 3-hydroxyisobutyrate dehydrogenase activity. Additionally, the cell can include, CoA hydrolase activity, poly hydroxyacid synthase activity, and/or lipase or esterase activity.

30 In another example, a cell including alanine 2,3-aminomutase activity; CoA transferase

activity; beta-alanyl-CoA ammonia lyase activity; alanine dehydrogenase or pyruvate-glutamate transaminase activity, and 3-HP-CoA dehydratase activity, produces a product, for example, 3-HP, and/or an ester of 3-HP, such as methyl 3-hydroxypropionate, ethyl 3-hydroxypropionate, propyl 3-hydroxypropionate, and/or butyl 3-hydroxypropionate. In some examples, the cell further includes 5 glutamate dehydrogenase activity, CoA transferase activity, 3-hydroxypropionyl-CoA hydrolase, and/or 3-hydroxyisobutyryl-CoA hydrolase activity. Accordingly, the disclosure also provides methods of producing one or more of these products. These methods involve culturing the cell that includes CoA transferase activity; beta-alanyl-CoA ammonia lyase activity; 3-HP-CoA dehydratase activity and in some examples glutamate dehydrogenase activity, CoA transferase activity, 3-10 hydroxypropionyl-CoA hydrolase, 3-hydroxyisobutryl-CoA hydrolase activity, and/or alanine dehydrogenase or pyruvate-glutamate transaminase activity, under conditions that allow the product to be produced. These cells also can include lipase or esterase activity.

Another aspect of the disclosure provides cells, which in addition to alanine 2,3-aminomutase activity, have CoA transferase activity; beta-alanyl-CoA ammonia lyase activity; 3-15 hydroxypropionyl-CoA dehydratase activity; and poly hydroxyacid synthase activity. In some examples, these cells can contain an exogenous nucleic acid molecule that encodes one or more polypeptides having: CoA transferase activity; beta-alanyl-CoA ammonia lyase activity; 3-hydroxypropionyl-CoA dehydratase activity; alanine dehydrogenase or pyruvate-glutamate transaminase activity, and poly hydroxyacid synthase activity. This cell can be used, for example, to 20 produce products such as polymerized 3-HP and co-polymers of 3-HP and other compounds such as butyrates, valerates and other compounds.

In another example, the cell, which in addition to alanine 2,3-aminomutase activity, has CoA transferase activity; beta-alanyl-CoA ammonia lyase activity; alanine dehydrogenase or pyruvate-glutamate transaminase activity, and poly hydroxyacid synthase activity, which can produce a 25 product, for example, polymerized 3-HP. In some examples, these cells can contain one or more exogenous nucleic acid molecules that encode one or more of polypeptides having CoA transferase activity; beta-alanyl-CoA ammonia lyase activity; and/or poly hydroxyacid synthase activity.

Another aspect of the disclosure provides a cell including alanine 2,3-aminomutase activity, CoA transferase activity, beta-alanyl-CoA ammonia lyase activity, alanine dehydrogenase or 30 pyruvate-glutamate transaminase activity, and lipase or esterase activity. In one example, the cell also includes CoA hydrolase activity. In some examples, the cell contains an exogenous nucleic acid molecule that encodes one or more polypeptides having CoA transferase activity; beta-alanyl-CoA ammonia lyase activity; lipase or esterase activity and/or CoA hydrolase activity. This cell can be 35 used, among other things, to produce products such as esters of acrylate (e.g., methyl acrylate, ethyl acrylate, propyl acrylate, and butyl acrylate).

Cells which can produce 1,3-propanediol, and methods of their use are disclosed. 1,3-propanediol can be generated from either 3-HP-CoA or 3-HP via the use of polypeptides having enzymatic activity. When converting 3-HP-CoA to 1,3-propanediol, polypeptides having

oxidoreductase activity or reductase activity, such as polypeptides having acetylating aldehyde:NAD(+) oxidoreductase and alcohol:NAD(+) oxidoreductase activities (e.g., enzymes from the 1.1.1.1 and/or 1.2.1.10 class of enzymes) can be used. When making 1,3-propanediol from 3-HP, a combination of a polypeptide having aldehyde dehydrogenase activity (e.g., an enzyme from the 5 1.2.2- class) and a polypeptide having alcohol dehydrogenase activity (e.g., an enzyme from the 1.1.1.- class) can be used, such as aldehyde dehydrogenase (NAD(P)+) (EC 1.2.1.-) and alcohol dehydrogenase (EC 1.1.1.1).

In some examples, products are produced *in vitro* (outside of a cell). In other examples, products are produced using a combination of *in vitro* and *in vivo* (within a cell) methods. In yet 10 other examples, products are produced *in vivo*. For methods involving *in vivo* steps, the cells can be isolated cultured cells or whole organisms such as transgenic plants, non-human mammals, or single-celled organisms such as yeast and bacteria (e.g., *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells). Hereinafter such cells are referred to as production cells. Products produced by these production cells can be organic products such as beta-alanine, 3-HP, pantothenate, and 15 derivatives thereof such as organic acids, polyols (i.e. 1,3-propanediol), coenzyme A (CoA), as well as an alanine 2,3-aminomutase described herein.

Pantothenate, a vitamin essential to many animals for growth and health, is involved in fatty acid synthesis and degradation. Deficiency of the vitamin results in generalized malaise clinically. Therefore, pantothenate produced using the methods disclosed herein can be administered to a subject 20 having a pantothenic deficiency, at a therapeutically effective dose. Cells that produce pantothenate, and methods of producing pantothenate from beta-alanine using the disclosed cells, are disclosed. Production cells used to produce pantothenate and/or CoA, can be used to express alpha-ketopantoate hydroxymethyltransferase (E.C. 2.1.2.11), alpha-ketopantoate reductase (E.C. 1.1.1.169), and 25 pantothenate synthase (E.C. 6.3.2.1), to produce pantothenate, or in addition pantothenate kinase (E.C. 2.7.1.33), 4'-phosphopantethenyl-1-cysteine synthetase (E.C. 6.3.2.5), 4'-phosphopantethenylcysteine decarboxylase (E.C. 4.1.1.36), ATP:4'-phosphopantetheine 30 adenyltransferase (E.C. 2.7.7.3), and dephospho-CoA kinase (E.C. 2.7.1.24), to produce coenzyme A.

BRIEF DESCRIPTION OF THE FIGURES

30 **FIG. 1** is a diagram of a pathway for generating 3-HP and derivatives thereof via a beta-alanine intermediate, and for making beta-alanine from alpha-alanine.

FIG. 2 is a diagram of a pathway for generating beta-alanine.

FIG. 3 is a diagram of a pathway for generating coenzyme A and pantothenate from beta-alanine.

35 **FIG. 4** is an alignment of a *B. subtilis* wild-type lysine 2,3-aminomutase (KAM, SEQ ID NO: 31), and a mutated form thereof which encodes an alanine 2,3-aminomutase (SEQ ID NO: 21). Substitutions are shown in bold. The Fe-S cluster -binding motif is underlined, and the putative PLP-binding motif is italicized.

- 6 -

FIG. 5 is an alignment of *P. gingivalis* wild-type lysine 2,3-aminomutase (kam, SEQ ID NO: 28) and a mutated form thereof which encodes an alanine 2,3-aminomutase (aam, SEQ ID NO: 30). Substitutions are shown in bold. The Fe-S cluster-binding motif is underlined, and the putative PLP-binding motif is italicized.

5 **FIG. 6** is an alignment of a *B. subtilis* and *P. gingivalis* wild-type lysine 2,3-aminomutase (kam, SEQ ID NOS: 31 and 28), and a mutated form thereof which encodes an alanine 2,3-aminomutase (aam, SEQ ID NOS: 21 and 29). Substitutions with a common location are in bold.

SEQUENCE LISTING

10 The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

15 **SEQ ID NOS: 1 and 2** are PCR primers used to clone a *B. subtilis* lysine 2,3-aminomutase (*KAM*) gene.

SEQ ID NO: 3 is a nucleic acid sequence of a *B. subtilis* *KAM* gene.

SEQ ID NOS: 4 and 5 are PCR primers used to amplify a *CAT* gene of pKD3.

20 **SEQ ID NOS: 6 and 7** are PCR primers used to confirm correct insertion of the *CAT* gene into the *panD* locus.

SEQ ID NOS: 8 and 9 are nucleic acid sequences of primers used to amplify the *CAT* gene of pKD3.

25 **SEQ ID NOS: 10 and 11** are nucleic acid sequences of primers used to generate an L103M mutation in the wildtype *B. subtilis* lysine 2,3-aminomutase gene.

SEQ ID NOS: 12 and 13 are nucleic acid sequences of primers used to generate an M136V mutation in the wildtype *B. subtilis* lysine 2,3-aminomutase gene.

30 **SEQ ID NOS: 14 and 15** are nucleic acid sequences of primers used to generate an D339H mutation in the wildtype *B. subtilis* lysine 2,3-aminomutase gene.

SEQ ID NOS: 16-19, 26, 27 and 32 are nucleic acid sequences of primers used to clone a 3-HP dehydrogenase gene from *Alcaligenes faecalis* M3A.

35 **SEQ ID NO: 20** is a nucleic acid sequence of an alanine 2,3-aminomutase DNA.

SEQ ID NO: 21 is an amino acid sequence of an alanine 2,3-aminomutase protein.

SEQ ID NO: 22 is a nucleic acid sequence of a beta-alanyl-CoA ammonia lyase (ACL-1) cDNA.

SEQ ID NO: 23 is an amino acid sequence of a beta-alanyl-CoA ammonia lyase (ACL-1) protein.

SEQ ID NO: 24 is a nucleic acid sequence of a CoA transferase cDNA.

SEQ ID NO: 25 is an amino acid sequence of a CoA transferase protein.

SEQ ID NO: 28 is an amino acid sequence of a *P. gingivalis* KAM.

- 7 -

SEQ ID NO: 29 is a nucleic acid sequence of an alanine 2,3-aminomutase.

SEQ ID NO: 30 is an amino acid sequence of an alanine 2,3-aminomutase protein.

SEQ ID NO: 31 is an amino acid sequence of a *B. subtilis* KAM.

SEQ ID NO: 33 is an nucleic acid sequence of a 3-HP dehydrogenase gene from 5 *Alcaligenes faecalis* M3A.

SEQ ID NO: 34 is an amino acid sequence of a 3-HP dehydrogenase gene from *Alcaligenes faecalis* M3A.

SEQ ID NOS: 35-37 are nucleic acid sequences of primers used to clone beta-alanine-CoA ammonia lyase (ACL-1 and ACL-2).

10 **SEQ ID NOS: 38-40** are nucleic acid sequences of primers used to clone CoA transferase from *E. coli*.

SEQ ID NOS: 41-48 are nucleic acid sequences of primers used to generate operons 1 and 2 which include ACL-1 or ACL-2, CoA transferase and CoA hydratase genes.

15 **SEQ ID NOS: 49-52** are nucleic acid sequences of primers used to generate operon 3 which includes 4-aminobutyrate aminotransferase and 3-hydroxyisobutyrate dehydrogenase genes.

SEQ ID NO: 53 is a nucleic acid sequence of a beta-alanyl-CoA ammonia lyase (ACL-2) cDNA.

SEQ ID NO: 54 is an amino acid sequence of a beta-alanyl-CoA ammonia lyase (ACL-2) protein.

20 **SEQ ID NOS: 55-56** are nucleic acid sequences of primers used to amplify the ATH-2 operon from the pATH-2-2-1 plasmid.

SEQ ID NOS: 57-58 are nucleic acid sequences of primers used to amplify the ATD operon from the pATD plasmid.

25 **SEQ ID NOS: 59-60** are nucleic acid sequences of primers used to amplify a *B. subtilis* alanine 2,3 aminomutase.

SEQ ID NOS: 61-62 are nucleic acid sequences of primers used to amplify a rat beta-alanine aminotransferase gene.

SEQ ID NOS: 63-64 are nucleic acid sequences of primers used to amplify a 3-HP dehydrogenase from *A. faecalis*.

30 **SEQ ID NOS: 65-66** are nucleic acid sequences of primers used to amplify an alpha-alanine aminotransferase gene from rat.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

Abbreviations and Terms

35 The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. As used herein, "comprising" means "including" and the singular forms "a" or "an" or "the" include plural references unless the context clearly dictates otherwise. For example, reference to "comprising

a protein" includes one or a plurality of such proteins, and reference to "comprising the cell" includes reference to one or more cells and equivalents thereof known to those skilled in the art, and so forth.

Unless explained otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs.

5 Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting. Other features and advantages of the disclosure are apparent from the following detailed description and the claims.

10 **Alanine 2,3-aminomutase:** An enzyme which can convert alpha-alanine to beta-alanine, for example in a cell. Includes any alanine 2,3-aminomutase gene, cDNA, RNA, or protein from any organism, such as a prokaryote. In one example, an alanine 2,3-aminomutase is a mutated lysine 2,3-aminomutase or a mutated lysine 5,6-aminomutase which has alanine 2,3-aminomutase activity.

15 Lysine 2,3-aminomutases (or genes annotated in genetic databases as lysine 2,3 aminomutase) can be obtained from any organism, such as a prokaryote, for example *Bacillus subtilis*, *Deinococcus radiodurans*, *Clostridium subterminale*, *Porphyromonas gingivalis* or *E. coli*, and mutated using any method known in the art.

20 In particular examples, an alanine 2,3-aminomutase nucleic acid sequence includes the sequence shown in SEQ ID NOS: 20 or 29, or fragments, variants, or fusions thereof that retain the ability to encode a peptide or protein having alanine 2,3-aminomutase activity. In another example, an alanine 2,3-aminomutase protein includes the amino acid sequence shown in SEQ ID NO: 21 or 30, or fragments, fusions, or variants thereof that retain alanine 2,3-aminomutase activity.

25 In another example, an alanine 2,3-aminomutase sequence includes a full-length wild-type sequence, such as SEQ ID NO: 21 or 30, as well as shorter sequences which retain the ability to convert alpha-alanine to beta-alanine, such as amino acids 50-390 of SEQ ID NO: 21, amino acids 101-339 of SEQ ID NO: 21, amino acids 15-390 of SEQ ID NO: 30, and amino acids 15-340 of SEQ ID NO 30. This description includes alanine 2,3-aminomutase allelic variants, as well as any variant, fragment, or fusion sequence which retains the ability to convert alpha-alanine to beta-alanine.

30 **Alanine 2,3-aminomutase activity:** The ability of an alanine 2,3-aminomutase to convert alpha-alanine to beta-alanine. In one example, such activity occurs in a cell. In another example, such activity occurs *in vitro*. Such activity can be measured using any assay known in the art, for example the screening assays and enzyme assays described in EXAMPLES 6 and 9-11. In addition, an enzyme with alanine 2,3-aminomutase activity can be identified by incubating the enzyme with either alpha-alanine or beta-alanine and determining the reaction products by high-performance liquid chromatography (for example using the method of , Abe *et al. J. Chromatography B*, 712:43-9, 1998). In one example, it is the ability of an alanine 2,3-aminomutase to convert alpha-alanine to beta-alanine in an *E. coli* mutant functionally deleted for the *panD* gene.

Antibody: A molecule including an antigen binding site which specifically binds (immunoreacts with) an antigen. Examples include polyclonal antibodies, monoclonal antibodies, humanized monoclonal antibodies, or immunologically effective portions thereof.

Includes immunoglobulin molecules and immunologically active portions thereof. Naturally occurring antibodies (e.g., IgG) include four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. However, the antigen-binding function of an antibody can be performed by fragments of a naturally occurring antibody. Immunologically effective portions of monoclonal antibodies include, but are not limited to: Fab, Fab', F(ab')₂, Fabc and Fv portions (for a review, see Better and Horowitz, *Methods. Enzymol.* 1989, 178:476-96). Other examples of antigen-binding fragments include, but are not limited to: (i) an Fab fragment consisting of the VL, VH, CL and CH1 domains; (ii) an Fd fragment consisting of the VH and CH1 domains; (iii) an Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (iv) a dAb fragment which consists of a VH domain; (v) an isolated complementarity determining region (CDR); and (vi) an F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region. Furthermore, although the two domains of the Fv fragment are coded for by separate genes, a synthetic linker can be made that enables them to be made as a single protein chain (known as single chain Fv (scFv) by recombinant methods. Such single chain antibodies are also included.

“Specifically binds” refers to the ability of a particular agent (a “specific binding agent”) to specifically react with a particular analyte, for example to specifically immunoreact with an antibody, or to specifically bind to a particular peptide sequence. The binding is a non-random binding reaction, for example between an antibody molecule and an antigenic determinant. Binding specificity of an antibody is typically determined from the reference point of the ability of the antibody to differentially bind the specific antigen and an unrelated antigen, and therefore distinguish between two different antigens, particularly where the two antigens have unique epitopes. An antibody that specifically binds to a particular epitope is referred to as a “specific antibody”.

Monoclonal or polyclonal antibodies can be produced to an alanine 2,3-aminomutase polypeptide (such as SEQ ID NO: 21 and/or 30), fragments of an alanine 2,3-aminomutase polypeptide (such as amino acids 50-390 of SEQ ID NO: 21, for example amino acids 101-339 of SEQ ID NO: 21, or amino acids 15-390 of SEQ ID NO: 30, for example amino acids 15-331 of SEQ ID NO: 30), or variants, fusions, or fragments thereof. Optimally, antibodies raised against one or more epitopes on a polypeptide antigen will specifically detect that polypeptide. That is, antibodies raised against one particular polypeptide would recognize and bind that particular polypeptide, and would not substantially recognize or bind to other polypeptides. The determination that an antibody specifically binds to a particular polypeptide is made by any one of a number of standard immunoassay methods; for instance, Western blotting (See, e.g., Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989).

- 10 -

To determine that an antibody preparation (such as a preparation produced in a mouse against an alanine 2,3-aminomutase polypeptide, for example SEQ ID NO: 21 or 30) specifically detects the appropriate polypeptide (e.g., an alanine 2,3-aminomutase polypeptide) by Western blotting, total cellular protein can be extracted from cells and separated by SDS-polyacrylamide gel electrophoresis. The separated total cellular protein can then be transferred to a membrane (e.g., nitrocellulose), and the antibody preparation incubated with the membrane. After washing the membrane to remove non-specifically bound antibodies, the presence of specifically bound antibodies can be detected using an appropriate secondary antibody (e.g., an anti-mouse antibody) conjugated to an enzyme such as alkaline phosphatase since application of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium results in the production of a densely blue-colored compound by immuno-localized alkaline phosphatase.

Substantially pure polypeptides suitable for use as an immunogen can be obtained from transfected cells, transformed cells, or wild-type cells. Polypeptide concentrations in the final preparation can be adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms per milliliter. In addition, polypeptides ranging in size from full-length polypeptides to polypeptides having as few as nine amino acid residues can be utilized as immunogens. Such polypeptides can be produced in cell culture, can be chemically synthesized using standard methods, or can be obtained by cleaving large polypeptides into smaller polypeptides that can be purified. Polypeptides having as few as nine amino acid residues in length can be immunogenic when presented to an immune system in the context of a Major Histocompatibility Complex (MHC) molecule such as an MHC class I or MHC class II molecule. Accordingly, polypeptides having at least 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 900, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, or more consecutive amino acid residues of an alanine 2,3-aminomutase polypeptide can be used as immunogens for producing antibodies.

Monoclonal antibodies to any of the polypeptides disclosed herein can be prepared from murine hybridomas according to the classic method of Kohler & Milstein (*Nature* 256:495, 1975) or a derivative method thereof.

Polyclonal antiserum containing antibodies to the heterogeneous epitopes of any polypeptide disclosed herein can be prepared by immunizing suitable animals with the polypeptide (or fragment, fusion, or variant thereof), which can be unmodified or modified to enhance immunogenicity. An effective immunization protocol for rabbits can be found in Vaitukaitis *et al.* (*J. Clin. Endocrinol. Metab.* 33:988-91, 1971).

Antibody fragments can be used in place of whole antibodies and can be readily expressed in prokaryotic host cells. Methods of making and using immunologically effective portions of monoclonal antibodies, also referred to as "antibody fragments," are well known and include those described in Better & Horowitz (*Methods Enzymol.* 178:476-96, 1989), Glockshuber *et al.* (*Biochemistry* 29:1362-7, 1990), U.S. Patent No. 5,648,237 ("Expression of Functional Antibody

Fragments"), U.S. Patent No. 4,946,778 ("Single Polypeptide Chain Binding Molecules"), U.S. Patent No. 5,455,030 ("Immunotherapy Using Single Chain Polypeptide Binding Molecules"), and references cited therein.

5 Antigen: A compound, composition, or substance that can stimulate the production of antibodies or a T-cell response in an animal, including compositions that are administered, such as injected or absorbed, to an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens. The term "antigen" includes all related antigenic epitopes.

10 cDNA (complementary DNA): A piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences which determine transcription. cDNA can be synthesized in the laboratory by reverse transcription from messenger RNA extracted from cells.

15 Conservative substitution: One or more amino acid substitutions (for example 1, 2, 5 or 10 residues) for amino acid residues having similar biochemical properties. Typically, conservative substitutions have little to no impact on the activity of a resulting polypeptide. For example, a conservative substitution is an amino acid substitution in an alanine 2,3-aminomutase peptide that does not substantially affect the ability of the peptide to convert alpha-alanine to beta-alanine. In a particular example, a conservative substitution is an amino acid substitution in an alanine 2,3-aminomutase peptide, such as a conservative substitution in SEQ ID NO: 21 or 30, that does not significantly alter the ability of the protein to convert alpha-alanine to beta-alanine. Methods that can be used to determine alanine 2,3-aminomutase activity are disclosed herein (EXAMPLES 6 and 9-11). An alanine scan can be used to identify which amino acid residues in an alanine 2,3-aminomutase peptide can tolerate an amino acid substitution. In one example, alanine 2,3-aminomutase activity is not altered by more than 25%, for example not more than 20%, for example not more than 10%, when an alanine, or other conservative amino acid (such as those listed below), is substituted for one or more native amino acids.

25 In one example, one conservative substitution is included in the peptide, such as a conservative substitution in SEQ ID NO: 21 or 30. In another example, 10 or less conservative substitutions are included in the peptide, such as five or less. A polypeptide can be produced to contain one or more conservative substitutions by manipulating the nucleotide sequence that encodes that polypeptide using, for example, standard procedures such as site-directed mutagenesis or PCR. Alternatively, a polypeptide can be produced to contain one or more conservative substitutions by using standard peptide synthesis methods.

30 Substitutional variants are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. Examples of amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions include: Ser for Ala; Lys for Arg; Gln or His for Asn; Glu for Asp; Ser for Cys; Asn for Gln; Asp for Glu; Pro for Gly; Asn or Gln for His; Leu or Val for Ile; Ile or Val for Leu; Arg or Gln

for Lys; Leu or Ile for Met; Met, Leu or Tyr for Phe; Thr for Ser; Ser for Thr; Tyr for Trp; Trp or Phe for Tyr; and Ile or Leu for Val.

Further information about conservative substitutions can be found in, among other locations in, Ben-Bassat *et al.*, (*J. Bacteriol.* 169:751-7, 1987), O'Regan *et al.*, (*Gene* 77:237-51, 1989), Sahin-Toth *et al.*, (*Protein Sci.* 3:240-7, 1994), Hochuli *et al.*, (*Bio/Technology* 6:1321-5, 1988), WO 00/67796 (Curd *et al.*) and in standard textbooks of genetics and molecular biology.

Deletion: The removal of a sequence of a nucleic acid, for example DNA, the regions on either side being joined together.

10 Detectable: Capable of having an existence or presence ascertained. For example, production of beta-alanine from alpha-alanine is detectable if the signal generated from the beta-alanine is strong enough to be measurable.

15 DNA: Deoxyribonucleic acid. DNA is a long chain polymer which comprises the genetic material of most living organisms (some viruses have genes comprising ribonucleic acid, RNA). The repeating units in DNA polymers are four different nucleotides, each of which comprises one of the four bases, adenine, guanine, cytosine and thymine bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides, referred to as codons, in DNA molecules code for amino acid in a polypeptide. The term codon is also used for the corresponding (and complementary) sequences of three nucleotides in the mRNA into which the DNA sequence is transcribed.

20 Exogenous: The term "exogenous" as used herein with reference to nucleic acid and a particular cell refers to any nucleic acid that does not originate from that particular cell as found in nature. Thus, a non-naturally-occurring nucleic acid is considered to be exogenous to a cell once introduced into the cell. A nucleic acid that is naturally-occurring also can be exogenous to a particular cell. For example, an entire chromosome isolated from a cell of person X is an exogenous nucleic acid with respect to a cell of person Y once that chromosome is introduced into Y's cell.

30 Functional deletion: A mutation, partial or complete deletion, insertion, or other variation made to a gene sequence which inhibits production of the gene product, and/or renders the gene product non-functional. For example, functional deletion of *panD* in *E. coli* prevents the production of β-alanine from aspartate by aspartate decarboxylase, which is encoded by the *panD* gene. This functional deletion of *panD* in *E. coli* inactivates aspartate decarboxylase which results in growth inhibition of the *E. coli* in the absence of beta-alanine or pantothenate in the growth medium.

35 Functionally Equivalent: Having an equivalent function. In the context of a alanine 2,3-aminomutase molecule, functionally equivalent molecules include different molecules that retain the function of alanine 2,3-aminomutase. For example, functional equivalents can be provided by sequence alterations in an alanine 2,3-aminomutase, wherein the peptide with one or more sequence alterations retains a function of the unaltered peptide, such that it retains its ability to convert alpha-alanine to beta-alanine.

Examples of sequence alterations include, but are not limited to, conservative substitutions, deletions, mutations, frameshifts, and insertions. In one example, a given polypeptide binds an antibody, and a functional equivalent is a polypeptide that binds the same antibody. Thus a functional equivalent includes peptides that have the same binding specificity as a polypeptide, and

5 that can be used as a reagent in place of the polypeptide (such as in the production of pantothenic acid and 3-HP). In one example a functional equivalent includes a polypeptide wherein the binding sequence is discontinuous, wherein the antibody binds a linear epitope. Thus, if the peptide sequence is MKNKWYKPKR (amino acids 1-10 of SEQ ID NO: 21) a functional equivalent includes discontinuous epitopes, that can appear as follows (**=any number of intervening amino acids): NH₂ -**.M**K**N**K**W**Y**K**P**K**R-COOH. In this example, the polypeptide is

10 functionally equivalent to amino acids 1-10 of SEQ ID NO: 21 if the three dimensional structure of the polypeptide is such that it can bind a monoclonal antibody that binds amino acids 1-10 of SEQ ID NO: 21.

15 **Hybridization:** A method of testing for complementarity in the nucleotide sequence of two nucleic acid molecules, based on the ability of complementary single-stranded DNA and/or RNA to form a duplex molecule. Nucleic acid hybridization techniques can be used to obtain an isolated nucleic acid within the scope of the disclosure. Briefly, any nucleic acid having some homology to an alanine 2,3-aminomutase (such as homology to SEQ ID NOS: 20 and 29 or variants or fragments thereof) can be used as a probe to identify a similar nucleic acid by hybridization under conditions of

20 moderate to high stringency. Once identified, the nucleic acid then can be purified, sequenced, and analyzed to determine if it is an alanine 2,3-aminomutase having alanine 2,3-aminomutase activity.

Hybridization can be done by Southern or Northern analysis to identify a DNA or RNA sequence, respectively, that hybridizes to a probe. The probe can be labeled, for example with a biotin, a fluorophore, digoxigenin, an enzyme, or a radioisotope such as ³²P. The DNA or RNA to be analyzed can be electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe using standard techniques well known in the art such as those described in sections 7.39-7.52 of Sambrook *et al.*, (1989) Molecular Cloning, second edition, Cold Spring Harbor Laboratory, Plainview, NY. Typically, a probe is at least about 20 nucleotides in length. For example, a probe including 20

30 contiguous nucleotides of an alanine 2,3-aminomutase (such as 20 contiguous nucleotides of SEQ ID NO: 20 or 29) can be used to identify an identical or similar nucleic acid. In addition, probes longer or shorter than 20 nucleotides can be used.

The disclosure also provides isolated nucleic acid sequences that are at least about 12 bases in length (e.g., at least about 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 100, 250, 500, 750, 35 1000, 1400, 2000, 3000, 4000, or 5000 bases in length) and hybridize, under hybridization conditions, to the sense or antisense strand of an alanine 2,3-aminomutase nucleic acid sequence, for example SEQ ID NO: 20 or 29). The hybridization conditions can be moderately or highly stringent hybridization conditions.

5 Moderately stringent hybridization conditions are when the hybridization is performed at about 42°C in a hybridization solution containing 25 mM KPO₄ (pH 7.4), 5X SSC, 5X Denhart's solution, 50 µg/mL denatured, sonicated salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about 5x10⁷ cpm/µg), while the washes are performed at about 50°C with a wash solution containing 2X SSC and 0.1% sodium dodecyl sulfate.

10 Highly stringent hybridization conditions are when the hybridization is performed at about 42°C in a hybridization solution containing 25 mM KPO₄ (pH 7.4), 5X SSC, 5X Denhart's solution, 50 µg/mL denatured, sonicated salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about 5x10⁷ cpm/µg), while the washes are performed at about 65°C with a wash solution containing 0.2X SSC and 0.1% sodium dodecyl sulfate.

15 Isolated: An "isolated" biological component (such as a nucleic acid molecule or protein) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids, proteins and peptides.

20 In one example, isolated refers to a naturally-occurring nucleic acid that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. For example, an isolated nucleic acid can be, without limitation, a recombinant DNA molecule of any length, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a recombinant DNA that exists as a separate molecule (e.g., 25 a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid sequence.

30 In one example, the term "isolated" as used with reference to nucleic acid also includes any non-naturally-occurring nucleic acid since non-naturally-occurring nucleic acid sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome. For example, non-naturally-occurring nucleic acid such as an engineered nucleic acid is considered to be isolated nucleic acid. Engineered nucleic acid can be made using common molecular cloning or 35 chemical nucleic acid synthesis techniques. Isolated non-naturally-occurring nucleic acid can be independent of other sequences, or incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or the genomic DNA of a prokaryote or

eukaryote. In addition, a non-naturally-occurring nucleic acid can include a nucleic acid molecule that is part of a hybrid or fusion nucleic acid sequence.

Leucine 2,3-aminomutase: An enzyme which can convert alpha-leucine to beta-leucine. Includes any leucine 2,3-aminomutase gene, cDNA, RNA, or protein from any organism, such as a 5 prokaryote or eukaryote, for example from rat, human, chicken, or *Clostridium sporogenes* (Poston, *J. Biol. Chem.* 251:1859-63, 1976). This description includes leucine 2,3-aminomutase allelic variants, as well as any variant, fragment, or fusion protein sequence which retains the ability to convert alpha-leucine to beta-leucine.

Lysine 2,3-aminomutase: An enzyme which can convert alpha-lysine to beta-lysine. 10 Includes any lysine 2,3-aminomutase gene, cDNA, RNA, or protein from any organism, such as a prokaryote, for example *Bacillus subtilis*, *Deinococcus radiodurans*, *Clostridium subterminale*, *Porphyromonas gingivalis*, *Aquifex aeolicus*, *Haemophilus influenzae*, or *E. coli*. This description includes lysine 2,3-aminomutase allelic variants, as well as any variant, fragment, or fusion sequence which retains the ability to convert alpha-lysine to beta-lysine. In one example, includes polypeptides 15 encoded by genes annotated as lysine 2,3-aminomutase in public DNA sequence databases, such as GenBank.

Nucleic acid: Encompasses both RNA and DNA including, without limitation, cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. The nucleic acid can be double-stranded or single-stranded. Where single-stranded, the nucleic acid can be the sense strand or the 20 antisense strand. In addition, nucleic acid can be circular or linear.

Oligonucleotide: A linear polynucleotide (such as DNA or RNA) sequence of at least 9 nucleotides, for example at least 15, 18, 24, 25, 27, 30, 50, 100 or even 200 nucleotides long.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second 25 nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

ORF (open reading frame): A series of nucleotide triplets (codons) coding for amino acids 30 without any termination codons. These sequences are usually translatable into a peptide.

Pantothenate or Pantothenic Acid: A commercially significant vitamin which is used in cosmetics, medicine, and nourishment. The terms pantothenic acid and pantothenate are used interchangeably herein, and refer not only to the free acid but also to the salts of D-pantothenic acid, such as the calcium salt, sodium salt, ammonium salt or potassium salt. Pantothenate can be 35 produced by chemical synthesis or biotechnologically from beta-alanine using the cells and methods disclosed herein.

Methods for measuring the amount of pantothenate are known (for example see U.S. Patent No. 6,184,006 to Rieping *et al.* and U.S. Patent No. 6,177,264 to Eggeling *et al.*). For example, a

quantitative determination of D-pantothenate can be made by using the *Lactobacillus plantarum* pantothenate assay (test strain: *Lactobacillus plantarum* ATCC 8014, Cat. No. 3211-30-3; culture medium: Bacto pantothenate assay medium (DIFCO Laboratories, Michigan, USA), cat. No. 0604-15-3). This indicator strain can grow only in the presence of pantothenate in the indicated culture medium and displays a photometrically measurable, linear dependency of the growth on the concentration of pantothenate in the medium. The hemicalcium salt of pantothenate can be used for calibration (Sigma Catalog Number P 2250). The optical density can be determined at a wavelength of 580 nm.

Peptide Modifications: The present disclosure includes alanine 2,3-aminomutase peptides, as well as synthetic embodiments. In addition, analogues (non-peptide organic molecules), derivatives (chemically functionalized peptide molecules obtained starting with the disclosed peptide sequences) and variants (homologs) having alanine 2,3-aminomutase activity can be utilized in the methods described herein. The peptides disclosed herein include a sequence of amino acids, that can be either L- and/or D- amino acids, naturally occurring and otherwise.

Peptides can be modified by a variety of chemical techniques to produce derivatives having essentially the same activity as the unmodified peptides, and optionally having other desirable properties. For example, carboxylic acid groups of the protein, whether carboxyl-terminal or side chain, may be provided in the form of a salt of a pharmaceutically-acceptable cation or esterified to form a C₁-C₁₆ ester, or converted to an amide of formula NR₁R₂ wherein R₁ and R₂ are each independently H or C₁-C₁₆ alkyl, or combined to form a heterocyclic ring, such as a 5- or 6-membered ring. Amino groups of the peptide, whether amino-terminal or side chain, may be in the form of a pharmaceutically-acceptable acid addition salt, such as the HCl, HBr, acetic, benzoic, toluene sulfonic, maleic, tartaric and other organic salts, or may be modified to C₁-C₁₆ alkyl or dialkyl amino or further converted to an amide.

Hydroxyl groups of the peptide side chains may be converted to C₁-C₁₆ alkoxy or to a C₁-C₁₆ ester using well-recognized techniques. Phenyl and phenolic rings of the peptide side chains may be substituted with one or more halogen atoms, such as F, Cl, Br or I, or with C₁-C₁₆ alkyl, C₁-C₁₆ alkoxy, carboxylic acids and esters thereof, or amides of such carboxylic acids. Methylene groups of the peptide side chains can be extended to homologous C₂-C₄ alkynes. Thiols can be protected with any one of a number of well-recognized protecting groups, such as acetamide groups. Those skilled in the art will also recognize methods for introducing cyclic structures into the peptides of this disclosure to select and provide conformational constraints to the structure that result in enhanced stability. For example, a C- or N-terminal cysteine can be added to the peptide, so that when oxidized the peptide will contain a disulfide bond, generating a cyclic peptide. Other peptide cyclizing methods include the formation of thioethers and carboxyl- and amino-terminal amides and esters.

Peptidomimetic and organomimetic embodiments are also within the scope of the present disclosure, whereby the three-dimensional arrangement of the chemical constituents of such peptido-

and organomimetics mimic the three-dimensional arrangement of the peptide backbone and component amino acid side chains, resulting in such peptido- and organomimetics of the proteins of this invention having detectable alanine 2,3-aminomutase activity. For computer modeling applications, a pharmacophore is an idealized, three-dimensional definition of the structural requirements for biological activity. Peptido- and organomimetics can be designed to fit each pharmacophore with current computer modeling software (using computer assisted drug design or CADD). See Walters, "Computer-Assisted Modeling of Drugs", in Klegerman & Groves, eds., 1993, *Pharmaceutical Biotechnology*, Interpharm Press: Buffalo Grove, IL, pp. 165-174 and *Principles of Pharmacology* Munson (ed.) 1995, Ch. 102, for descriptions of techniques used in CADD. Also included within the scope of the disclosure are mimetics prepared using such techniques. In one example, a mimetic mimics the alanine 2,3-aminomutase activity generated by an alanine 2,3-aminomutase or a variant, fragment, or fusion thereof.

15 Polynucleotide: A linear nucleic acid sequence of any length. Therefore, a polynucleotide includes molecules which are at least about 15, 25, 50, 75, 100, 200 or 400 (oligonucleotides) and also nucleotides as long as a full-length cDNA.

20 Probes and primers: A "probe" includes an isolated nucleic acid containing a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, fluorophores, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed in, for example, Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual* 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, and Ausubel *et al.* (ed.) *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987.

25 "Primers" are typically nucleic acid molecules having ten or more nucleotides (e.g., nucleic acid molecules having between about 10 nucleotides and about 100 nucleotides). A primer can be annealed to a complementary target nucleic acid strand by nucleic acid hybridization to form a hybrid between the primer and the target nucleic acid strand, and then extended along the target nucleic acid strand by, for example, a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, for example, by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods.

30 Methods for preparing and using probes and primers are described, for example, in references such as Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; Ausubel *et al.* (ed.), *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987; and Innis *et al.*, *PCR Protocols: A Guide to Methods and Applications*, Academic Press: San Diego, 1990. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.). One of skill in the art will appreciate that the specificity of a particular probe or primer increases with the length, but that a probe or primer can range in size from a

full-length sequence to sequences as short as five consecutive nucleotides. Thus, for example, a primer of 20 consecutive nucleotides can anneal to a target with a higher specificity than a corresponding primer of only 15 nucleotides. Thus, in order to obtain greater specificity, probes and primers can be selected that comprise, for example, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 5 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, or more consecutive nucleotides.

Promoter: An array of nucleic acid control sequences which direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes 10 distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide preparation is one in which the peptide or protein is more enriched than the peptide or protein is in its environment within a cell, such that the peptide is 15 substantially separated from cellular components (nucleic acids, lipids, carbohydrates, and other polypeptides) that may accompany it. In another example, a purified peptide preparation is one in which the peptide is substantially-free from contaminants, such as those that might be present following chemical synthesis of the peptide.

In one example, an alanine 2,3-aminomutase peptide is purified when at least 50% by weight 20 of a sample is composed of the peptide, for example when at least 60%, 70%, 80%, 85%, 90%, 92%, 95%, 98%, or 99% or more of a sample is composed of the peptide. Examples of methods that can be used to purify an antigen, include, but are not limited to the methods disclosed in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, Ch. 17). Protein purity can be determined by, for example, polyacrylamide gel electrophoresis of a protein sample, 25 followed by visualization of a single polypeptide band upon staining the polyacrylamide gel; high-pressure liquid chromatography; sequencing; or other conventional methods.

Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring and/or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, 30 more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Recombinant is also used to describe nucleic acid molecules that have been artificially manipulated, but contain the same regulatory sequences and coding regions that are found in the organism from which the nucleic acid was isolated.

Sequence identity/similarity: The identity/similarity between two or more nucleic acid 35 sequences, or two or more amino acid sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the

percentage, the more similar the sequences are. Homologs or orthologs of nucleic acid or amino acid sequences possess a relatively high degree of sequence identity/similarity when aligned using standard methods. This homology is more significant when the orthologous proteins or cDNAs are derived from species which are more closely related (e.g., human and mouse sequences), compared to species more distantly related (e.g., human and *C. elegans* sequences).

5 Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins & Sharp, *Gene*, 73:237-44, 1988; Higgins & Sharp, *CABIOS* 5:151-3, 1989; Corpet *et al.*, *Nuc. Acids Res.* 16:10881-90, 1988; Huang *et al.* *Computer Appl. in the Biosciences* 8, 155-65, 1992; and Pearson *et al.*, *Meth. Mol. Bio.* 24:307-31, 1994. Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

10 The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

15 BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. To compare two nucleic acid sequences, the options can be set as follows: -i is set to a file containing the first nucleic acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second nucleic acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastn; -o is set to any desired file name (e.g., C:\output.txt); -q is set to -1; -r is set to 2; and all other options are left at their default setting. For example, the following command can be used to generate 20 an output file containing a comparison between two sequences: C:\BL2seq -i c:\seq1.txt -j c:\seq2.txt -p blastn -o c:\output.txt -q -1 -r 2.

25 To compare two amino acid sequences, the options of BL2seq can be set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison 30 between two amino acid sequences: C:\BL2seq -i c:\seq1.txt -j c:\seq2.txt -p blastp -o c:\output.txt. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, 35 then the designated output file will not present aligned sequences.

Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is presented in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the

sequence set forth in the identified sequence, or by an articulated length (e.g., 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a nucleic acid sequence that has 1166 matches when aligned with a test sequence having 1154 nucleotides is 75.0 percent identical to the test sequence (i.e., $1166 \div 1154 \times 100 = 75.0$). The percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 are rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 are rounded up to 75.2. The length value will always be an integer. In another example, a target sequence containing a 20-nucleotide region that aligns with 20 consecutive nucleotides from an identified sequence as follows contains a region that shares 75 percent sequence identity to that identified sequence (i.e., $15 \div 20 \times 100 = 75$).

Target Sequence:	AGGTCGTGTACTGTCAGTCA	1	20
Identified Sequence:	ACGTGGTGAAC TGCCAGTGA		

15 For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). Homologs are typically characterized by possession of at least 70% sequence identity counted over the full-length alignment with an amino acid sequence using the NCBI Basic Blast 2.0, gapped blastp with databases such as the nr or swissprot database. Queries searched with the blastn program are filtered with DUST (Hancock and Armstrong, 1994, *Comput. Appl. Biosci.* 10:67-70). Other programs use SEG. In addition, a manual alignment can be performed. Proteins with even greater similarity will show increasing percentage identities when assessed by this method, such as at least 75%, 80%, 85%, 90%, 95%, or 99% sequence identity.

When aligning short peptides (fewer than around 30 amino acids), the alignment should be performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). Proteins with even greater similarity to the reference sequence will show increasing percentage identities when assessed by this method, such as at least 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% sequence identity. When less than the entire sequence is being compared for sequence identity, homologs will typically possess at least 75% sequence identity over short windows of 10-20 amino acids, and can possess sequence identities of at least 85%, 90%, 95% or 98% depending on their identity to the reference sequence. Methods for determining sequence identity over such short windows are described at the NCBI web site.

One indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence-dependent and are different under different environmental parameters. Nucleic acid molecules that hybridize under stringent conditions to an *alanine 2,3-aminomutase* gene sequence typically hybridize to a probe based on either an entire *alanine 2,3-aminomutase* gene or selected portions of the gene, respectively, under conditions described above.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode identical or similar (conserved) amino acid sequences, due to the degeneracy of the genetic code. Changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid molecules that all encode substantially the same protein. Such homologous nucleic acid sequences can, 5 for example, possess at least 60%, 70%, 80%, 90%, 95%, 98%, or 99% sequence identity determined by this method.

One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is possible that strongly significant homologs could be obtained that fall outside the ranges provided.

10 An alternative (and not necessarily cumulative) indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

15 **Specific binding agent:** An agent that binds substantially only to a defined target, such as a peptide target. For example, an alanine 2,3-aminomutase binding agent includes anti-alanine 2,3-aminomutase antibodies and other agents (such as peptide or drugs) that bind substantially to only an alanine 2,3-aminomutase. Antibodies to an alanine 2,3-aminomutase protein (or fragments thereof) can be used to purify or identify such a protein.

20 **Transformed:** A cell into which a nucleic acid molecule has been introduced, for example by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including, but not limited to transfection with viral vectors, conjugation, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

25 **Variants, fragments or fusion proteins:** The disclosed alanine 2,3 aminomutase proteins, include variants, fragments, and fusions thereof. DNA sequences which encode for a protein (for example SEQ ID NO: 20 or 29), fusion alanine 2,3 aminomutase protein, or a fragment or variant of an alanine 2,3 aminomutase protein, can be engineered to allow the protein to be expressed in eukaryotic cells, bacteria, insects, and/or plants. To obtain expression, the DNA sequence can be altered and operably linked to other regulatory sequences. The final product, which contains the regulatory sequences and the protein, is referred to as a vector. This vector can be introduced into 30 eukaryotic, bacteria, insect, and/or plant cells. Once inside the cell the vector allows the protein to be produced.

35 A fusion protein including a protein, such as an alanine 2,3-aminomutase (or variant, polymorphism, mutant, or fragment thereof), for example SEQ ID NO: 21 or 30, linked to other amino acid sequences that do not inhibit the desired activity of alanine 2,3-aminomutase, for example the ability to convert alpha-alanine to beta-alanine. In one example, the other amino acid sequences are no more than about 10, 12, 15, 20, 25, 30, or 50 amino acids in length.

One of ordinary skill in the art will appreciate that a DNA sequence can be altered in numerous ways without affecting the biological activity of the encoded protein. For example, PCR

can be used to produce variations in the DNA sequence which encodes an alanine 2,3-aminomutase. Such variants can be variants optimized for codon preference in a host cell used to express the protein, or other sequence changes that facilitate expression.

5 **Vector:** A nucleic acid molecule as introduced into a cell, thereby producing a transformed cell. A vector may include nucleic acid sequences that permit it to replicate in the cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic elements known in the art.

Alanine 2,3-Aminomutase Nucleic Acids and Polypeptides

10 Polypeptides having alanine 2,3-aminomutase activity are disclosed herein. In one example, the polypeptide is a mutated aminomutase amino acid sequence, such as a lysine 2,3-aminomutase, leucine 2,3-aminomutase, or lysine 5,6-aminomutase sequence. Examples of a polypeptide having alanine 2,3-aminomutase activity are shown in SEQ ID NOS: 21 and 30. However, the disclosure also encompasses variants, fusions, and fragments of SEQ ID NOS: 21 and 30 which retain alanine 15 2,3-aminomutase activity. Examples of fragments which can be used include, but are not limited to: amino acids 50-390, 50-350, 60-350, 75-340, or 100-339 of SEQ ID NO: 21 and amino acids 1-390, 15-390, 15-340 or 19-331 of SEQ ID NO:30. Examples of substitutions which can be made, while still retaining alanine 2,3-aminomutase activity, include, but are not limited to: V21I or V21L; Y71P; L17I; K361R; A410V; and/or Y430F or Y430W of SEQ ID NO: 21, and T40S; V96I or V96L; 20 D102E; A252V; and/or L393V of SEQ ID NO: 30, as well as combinations thereof.

25 The disclosure provides enzyme polypeptides, such as an alanine 2,3-aminomutase (for example SEQ ID NO: 21 and/or 30, and variants, fragments, and fusions thereof that retain alanine 2,3-aminomutase activity). One skilled in the art will understand that variant enzyme sequences can be used, as long as the enzyme retains the desired enzyme activity, such as alanine 2,3-aminomutase 30 activity. For example, the disclosure provides polypeptides that contain at least 15 contiguous amino acids which are identical to an enzyme sequence, such as an alanine 2,3-aminomutase sequence. It will be appreciated that the disclosure also provides polypeptides that contain an amino acid sequence that is greater than at least 15 amino acid residues (e.g., at least 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 50, 75, 100, 150, 200, 250, 300 or more amino acid residues) and identical to any enzyme disclosed herein or otherwise publicly available.

35 In addition, the disclosure provides enzyme polypeptides, such as an alanine 2,3-aminomutase peptide (e.g. SEQ ID NO: 21 and/or 30), which includes an amino acid sequence having a variation of the enzyme amino acid sequence. Variant sequences can contain a single insertion, a single deletion, a single substitution, multiple insertions, multiple deletions, multiple substitutions, or any combination thereof (e.g., single deletion together with multiple insertions). Such polypeptides share at least 60, 65, 70, 75, 80, 85, 90, 95, 97, 98, or 99% sequence identity with an enzyme sequence, such as an alanine 2,3-aminomutase sequence, as long as the peptide encoded

by the amino acid sequence retains the desired enzyme activity.

Polypeptides having a variant amino acid sequence can retain enzymatic activity, such as alanine 2,3-aminomutase activity. Such polypeptides can be produced by manipulating the nucleotide sequence encoding a polypeptide using standard procedures such as site-directed 5 mutagenesis or PCR. One type of modification includes the substitution of one or more amino acid residues, such as no more than 10 amino acids, for amino acid residues having a similar biochemical property, that is, a conservative substitution.

More substantial changes can be obtained by selecting substitutions that are less 10 conservative, e.g., selecting residues that differ more significantly in their effect on maintaining: (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation; (b) the charge or hydrophobicity of the polypeptide at the target site; or (c) the bulk of the side chain. The substitutions that in general are expected to produce the greatest changes 15 in polypeptide function are those in which: (a) a hydrophilic residue, e.g., serine or threonine, is substituted for (or by) a hydrophobic residue, e.g., leucine, isoleucine, phenylalanine, valine or alanine; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an 20 electropositive side chain, e.g., lysine, arginine, or histidine, is substituted for (or by) an electronegative residue, e.g., glutamic acid or aspartic acid; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine. The effects of 25 these amino acid substitutions (or other deletions or additions) can be assessed for polypeptides having enzymatic activity by analyzing the ability of the polypeptide to catalyze the conversion of the same substrate as the related native polypeptide to the same product as the related native polypeptide. Accordingly, polypeptides having no more than 5, 10, 20, 30, 40, or 50 conservative substitutions are provided herein.

Also disclosed are isolated nucleic acids that encode polypeptides having alanine 2,3- 25 aminomutase activity, for example a sequence which includes SEQ ID NO: 20 or 29. However, the disclosure also encompasses variants, fusions, and fragments of SEQ ID NOS: 20 and 29 which retain the ability to encode a protein or peptide having alanine 2,3-aminomutase activity. In one example an isolated nucleic acid encoding a polypeptide having alanine 2,3-aminomutase activity is operably linked to a promoter sequence, and can be part of a vector. The nucleic acid can be a 30 recombinant nucleic acid, that can be used to transform cells and make transformed cells and/or transgenic non-human mammals.

Transformed cells including at least one exogenous nucleic acid molecule which encodes a 35 polypeptide having alanine 2,3-aminomutase activity (such as SEQ ID NO: 20 and/or 29 or fragments, fusions, or variants thereof that retain alanine 2,3-aminomutase activity), is disclosed. In one example, such a transformed cell produces beta-alanine from alpha-alanine. In another example, the cell produces 3-HP, pantothenate, CoA, and/or organic compounds such as 1,3-propanediol.

The nucleic acid sequences encoding the enzymes disclosed herein, such as alanine 2,3-aminomutase (SEQ ID NO: 20 and 29), lysine 2,3-aminomutase (SEQ ID NOS: 3 and 28), and beta-

5 alanyl-CoA ammonia lyase (SEQ ID NO: 22), (as well as any other enzyme disclosed herein), can contain an entire nucleic acid sequence encoding the enzyme, as well as a portions thereof that retain the desired enzyme activity. For example, an enzyme nucleic acid can contain at least 15 contiguous nucleotides of an enzyme nucleic acid sequence. It will be appreciated that the disclosure also provides isolated nucleic acid that contains a nucleotide sequence that is greater than 15 nucleotides (e.g., at least 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 75, 10, 200, 500 or more nucleotides) in length and identical to any portion of an enzyme sequence, such as an alanine 2,3-aminomutase sequence shown in SEQ ID NO: 20 and/or 29.

10 In addition, the disclosure provides isolated enzyme nucleic acid sequences which contains a variation of an enzyme sequence, such as a variant alanine 2,3-aminomutase nucleic acid sequence. Variants can contain a single insertion, a single deletion, a single substitution, multiple insertions, 15 multiple deletions, multiple substitutions, or any combination thereof (e.g., single deletion together with multiple insertions) as long as the peptide encoded thereby retains alanine 2,3-aminomutase activity. Such isolated nucleic acid molecules can share at least 60, 70, 75, 80, 85, 90, 92, 95, 97, 98, 20 or 99% sequence identity with an enzyme sequence, such as an alanine 2,3-aminomutase sequence, as long as the peptide encoded by the nucleic acid retains the desired enzyme activity, such as alanine 2,3-aminomutase activity. For example, the following variations can be made to the alanine 2,3-aminomutase nucleic acid sequence: for SEQ ID NO: 20, the "a" at position 12 can be substituted with an "g"; the "g" at position 1050 can be substituted with an "a"; the "a" at position 255; can be substituted with an "g" "t" or "c;" for SEQ ID NO: 29, the "a" at position 6 can be substituted with a "g" "t" or "c"; the "t" at position 66 can be substituted with a "c"; and the "g" at position 315; can be substituted with an "a" "t" or "c."

25 Codon preferences and codon usage tables for a particular species can be used to engineer isolated nucleic acid molecules that take advantage of the codon usage preferences of that particular species. For example, the enzymes disclosed herein can be designed to have codons that are preferentially used by a particular organism of interest.

30 The disclosure also provides isolated nucleic acid sequences that encode for an enzyme, such as alanine 2,3-aminomutase, wherein the sequence is at least about 12 bases in length (e.g., at least about 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 100, 250, 500, 750, 1000, 1500, 2000, 3000, 4000, or 5000 bases in length) and hybridizes, under hybridization conditions, to the sense or antisense strand of a nucleic acid encoding the enzyme. The hybridization conditions can be moderately or highly stringent hybridization conditions.

35 Polypeptides and nucleic acid encoding polypeptide can be produced by standard DNA mutagenesis techniques, for example, M13 primer mutagenesis. Details of these techniques are provided in Sambrook *et al.* (ed.), Molecular Cloning: A Laboratory Manual 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring, Harbor, N.Y., 1989, Ch. 15. Nucleic acid molecules can contain changes of a coding region to fit the codon usage bias of the particular organism into which the molecule is to be introduced.

Alternatively, the coding region can be altered by taking advantage of the degeneracy of the genetic code to alter the coding sequence in such a way that, while the nucleic acid sequence is substantially altered, it nevertheless encodes a polypeptide having an amino acid sequence identical or substantially similar to the native amino acid sequence. For example, because of the degeneracy of the genetic code, alanine is encoded by the four nucleotide codon triplets: GCT, GCA, GCC, and GCG. Thus, the nucleic acid sequence of the open reading frame can be changed at an alanine position to any of these codons without affecting the amino acid sequence of the encoded polypeptide or the characteristics of the polypeptide. Based upon the degeneracy of the genetic code, nucleic acid variants can be derived from a nucleic acid sequence using a standard DNA mutagenesis techniques as described herein, or by synthesis of nucleic acid sequences. Thus, this disclosure also encompasses nucleic acid molecules that encode the same polypeptide but vary in nucleic acid sequence by virtue of the degeneracy of the genetic code.

Cells with Alanine 2,3-Aminomutase Activity

Cells having alanine 2,3-aminomutase activity are disclosed. Such cells can produce beta-alanine from alpha-alanine. In one example, such cells have alanine 2,3-aminomutase activity due to a naturally occurring mutation, and/or a mutation induced in the chromosome(s) of the cell, for example by exposing the cell to chemical or UV mutagenesis. Cells including alanine 2,3-aminomutase activity can be eukaryotic or prokaryotic. Examples of such cells include, but are not limited to *Lactobacillus*, *Lactococcus*, *Bacillus*, *Escherichia*, *Geobacillus*, *Corynebacterium*, *Clostridium*, fungal, plant, and yeast cells. In one example, a plant cell is part of a plant, such as a transgenic plant.

In one example, cells having alanine 2,3-aminomutase activity are transformed cells. Such cells can include at least one exogenous nucleic acid molecule that encodes an alanine 2,3-aminomutase, for example a sequence comprising SEQ ID NO: 20 or 29, or variants, fragments, or fusions thereof that retain the ability to encode a protein having alanine 2,3-aminomutase activity. In one example, the exogenous nucleic acid molecule is a mutated lysine 2,3-aminomutase, such as a mutated prokaryotic lysine 2,3-aminomutase. In specific examples, the mutated prokaryotic lysine 2,3-aminomutase is a mutated *Bacillus subtilis*, *Deinococcus radiodurans*, *Clostridium subterminale*, *Aquifex aeolicus*, *Haemophilus influenzae*, *E. coli*, or *Porphyromonas gingivalis* lysine 2,3-aminomutase. Other lysine 2,3-aminomutases can be identified by using methods known in the art, for example by searching for similar sequences on BLAST and/or by using hybridization methods. In a specific example, the mutated lysine 2,3-aminomutase is a mutated *B. subtilis* or a mutated *P. gingivalis* lysine 2,3-aminomutase. In an another example, the exogenous nucleic acid molecule is a mutated lysine 5,6-aminomutase, such as a mutated prokaryotic lysine 5,6-aminomutase. Alternatively, the exogenous nucleic acid molecule is a mutated leucine 2,3-aminomutase, or a mutated lysine 5,6-aminomutase, such as a mutated *C. sticklandii* lysine 5,6-aminomutase.

In a particular example, the mutated lysine 2,3-aminomutase is a mutated *B. subtilis* lysine 2,3-aminomutase having a substitution at position L103, D339 and/or M136. For example, the substitution can include a L103M, L103K, L103R, L103E, or L103S substitution. In another or additional example, the substitution includes a D339H, D339Q, D339T, or D339N substitution. In 5 yet another example, the substitution can include a L103M, a M136V substitution, a D339H substitution, or any combination thereof.

Cells which include alanine 2,3-aminomutase activity as well as other enzyme activities, are disclosed. Such cells can be used to produce beta-alanine, 3-HP, pantothenate, CoA, and organic acids, polyols such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, 10 polymerized 3-HP, co-polymers of 3-HP and other compounds such as butyrates, valerates and other compounds, and esters of 3-HP.

In one example, such cells also include alanine dehydrogenase or pyruvate/glutamate transaminase activity, CoA transferase activity or CoA synthetase, beta-alanyl-CoA ammonia lyase activity, 3-HP-CoA dehydratase activity, glutamate dehydrogenase activity, 3-hydroxypropionyl-CoA 15 hydrolase, or 3-hydroxyisobutryl-CoA hydrolase activity. In another example, such cells also include alanine dehydrogenase or pyruvate-glutamate transaminase activity, 4-aminobutyrate and/or beta-alanine – 2-oxoglutarate aminotransferase activity, glutamate dehydrogenase activity, and 3-HP or 3-hydroxyisobutyrate dehydrogenase activity. In these examples, the cells can be used to produce 3-HP.

20 In another example, the cells also include alanine dehydrogenase or pyruvate/glutamate transaminase activity, CoA transferase or CoA synthetase activity, beta-alanyl-CoA ammonia lyase activity, 3-HP-CoA dehydratase activity, glutamate dehydrogenase activity, and 3-hydroxypropionyl-CoA hydrolase or 3-hydroxyisobutryl-CoA hydrolase activity. In another example, such cells also include alanine dehydrogenase or pyruvate-glutamate transaminase activity, 4-aminobutyrate and/or 25 beta-alanine–2-oxoglutarate aminotransferase activity, glutamate dehydrogenase activity, and 3-HP or 3-hydroxyisobutyrate dehydrogenase activity; and lipase or esterase activity. Such cells can be used to produce an ester of 3-HP, such as methyl 3-hydroxypropionate, ethyl 3-hydroxypropionate, propyl 3-hydroxypropionate, butyl 3-hydroxypropionate, or 2-ethylhexyl 3-hydroxypropionate.

30 In another example, the cells also include alanine dehydrogenase or pyruvate/glutamate transaminase activity, CoA synthetase activity, beta-alanyl-CoA ammonia lyase activity, 3-HP-CoA dehydratase activity, glutamate dehydrogenase activity; and poly hydroxacid synthase activity. Such cells can be used to produce polymerized 3-HP.

35 In yet another example, the cells also include alanine dehydrogenase or pyruvate/glutamate transaminase activity, CoA synthetase activity, beta-alanyl-CoA ammonia lyase activity, glutamate dehydrogenase activity and poly hydroxacid synthase activity. Such cells can be used to produce polymerized acrylate.

In another example, the cells also include alanine dehydrogenase or pyruvate/glutamate transaminase activity, CoA transferase or CoA synthetase activity, beta-alanyl-CoA ammonia lyase

activity, glutamate dehydrogenase activity, and lipase or esterase activity, wherein the cells can be used to produce an ester of acrylate, such as methyl acrylate, ethyl acrylate, propyl acrylate, or butyl acrylate.

Alternatively, such cells also include alanine dehydrogenase or pyruvate-glutamate 5 transaminase activity, CoA transferase or CoA synthetase activity, beta-alanyl-CoA ammonia lyase activity, 3-HP-CoA dehydratase activity, glutamate dehydrogenase activity, 3-hydroxypropionyl-CoA hydrolase or 3-hydroxyisobutryl-CoA hydrolase activity, and aldehyde or alcohol dehydrogenase activity. Such cells can be used to produce 1,3-propanediol.

In one example, the cells also have alpha-ketopantoate hydroxymethyltransferase (E.C. 10 2.1.2.11), alpha-ketopantoate reductase (E.C. 1.1.1.169), and pantothenate synthase (E.C. 6.3.2.1) activity. Such cells can be used to produce pantothenate. Alternatively or in addition, the cells also have pantothenate kinase (E.C. 2.7.1.33), 4'-phosphopantethenyl-1-cysteine synthetase (E.C. 15 6.3.2.5), 4'-phosphopantethenylcysteine decarboxylase (E.C. 4.1.1.36), ATP:4'-phosphopantetheine adenyltransferase (E.C. 2.7.7.3), and dephospho-CoA kinase (E.C. 2.7.1.24) activity. Such cells can be used to produce coenzyme A (CoA).

Methods to Identify Cells Having Alanine 2,3-Aminomutase Activity

A method of identifying a cell having alanine 2,3-aminomutase activity is disclosed. The method includes culturing a cell, such as a prokaryotic cell, which is functionally deleted for *panD*, in 20 media which includes alpha-alanine, but not beta-alanine or pantothenate, or in media in which the cell can produce alpha-alanine from media sources of carbon, oxygen, hydrogen, and nitrogen, but which does not include beta-alanine or pantothenate, and identifying cells capable of growing in the beta-alanine or pantothenate deficient-media. In particular examples, the cell is also functionally deleted for *panF*. Growth of the cell indicates that the cell is producing beta-alanine from alpha-alanine, which indicates the cell has alanine 2,3-aminomutase activity. In contrast, if a cell does not 25 grow and/or survive on the beta-alanine or pantothenate deficient-media, this indicates that the cell is not producing beta-alanine from alpha-alanine, which indicates the cell does not have alanine 2,3-aminomutase activity.

In one example, the cell functionally deleted for *panD* is transformed with one or more 30 mutated aminomutases, such as libraries including mutated lysine 2,3-aminomutase, mutated leucine 2,3-aminomutase, and/or mutated lysine 5,6-aminomutase. In a particular example, the cell is transformed with a library of mutated lysine 2,3-aminomutases, prior to culturing and screening the cells. The enzyme lysine 2,3-aminomutase has been previously cloned from *Clostridium subterminale* SB4 (Chirpich *et al.*, *J. Biol. Chem.* 245:1778-89, 1970) and *Bacillus subtilis* (Chen *et al.*, *Biochem. J.* 348:539-49, 2000), and has been shown to catalyze the interconversion of lysine and 35 beta-lysine. Mutant aminomutases, such as a mutant lysine 2,3-aminomutase, can be screened for their ability to confer alanine 2,3-aminomutase activity. In addition, although a polypeptide having alanine 2,3-aminomutase activity has not been previously described, such an enzyme may exist in

nature. Thus, a cell functionally deleted for *panD* can be transformed with a library including a gene encoding for alanine 2,3-aminomutase, and the gene isolated by its ability to confer growth to this cell in media containing alpha-alanine, or carbon, oxygen, hydrogen, and nitrogen sources such that the cell can generate alpha-alanine, but not containing beta-alanine or pantothenate.

5 In another example, the method further includes identifying a mutation in the mutated aminomutase(s) following identifying a cell which grows in the media, wherein the mutated aminomutase(s) confers alanine 2,3-aminomutase activity to the cell. To identify the mutation, the aminomutase nucleic acid or amino acid can be sequenced and compared to a non-mutated aminomutase sequence, to identify mutations that confer alanine 2,3-aminomutase activity to the cell.

10

Methods of Producing a Peptide having Alanine 2,3-Aminomutase Activity

A method for producing alanine 2,3-aminomutase peptides having alanine 2,3-aminomutase activity, is disclosed. The method includes culturing the disclosed cells having alanine 2,3-aminomutase activity under conditions that allow the cell to produce the alanine 2,3-aminomutase peptide. In one example, the method includes culturing cells having one or more exogenous nucleic acid molecules which encode for an alanine 2,3-aminomutase (such as a sequence which includes SEQ ID NO: 20 and/or 29 or variants, fusions, or fragments thereof that retain alanine 2,3-aminomutase activity), such that the alanine 2,3-aminomutase is produced.

20 A method for making beta-alanine from alpha-alanine is also disclosed. In one example, the method includes culturing the disclosed cells having alanine 2,3-aminomutase activity under conditions that allow the cell to produce beta-alanine from alpha-alanine. In one example, the method includes culturing cells having one or more exogenous nucleic acid molecules which encode for an alanine 2,3-aminomutase, such that the alanine 2,3-aminomutase is capable of producing beta-alanine from alpha-alanine. In one example, the exogenous nucleic acid is a sequence that includes SEQ ID NO: 20 and/or 29 or variants, fusions, or fragments thereof that retain alanine 2,3-aminomutase activity.

25 In particular examples, the cell is functionally deleted for *panD*, or *panD* and *panF*.

Pathways for Producing 3-HP, Pantothenate and Derivatives Thereof

30 Methods and materials related to producing beta-alanine from alpha-alanine, via an alanine 2,3-aminomutase, such as using the disclosed alanine 2,3-aminomutase sequences and the disclosed cells having alanine 2,3-aminomutase activity are disclosed. In addition, methods and materials related to producing pantothenate and 3-HP from beta-alanine, as well as CoA and organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, 35 polymerized 3-HP, co-polymers of 3-HP and other compounds such as butyrates, valerates and other compounds, and esters of 3-HP, are disclosed. Specifically, the disclosure provides alanine 2,3-aminomutase nucleic acids (such as SEQ ID NO: 20 and 29), polypeptides (such as SEQ ID NO: 21 and 30), host cells, and methods and materials for producing beta-alanine from alpha-alanine, which

can be used to more efficiently make beta-alanine pantothenate and 3-HP as well as derivatives thereof such as CoA and organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, and esters of 3-HP.

Several metabolic pathways can be used to produce organic compounds from beta-alanine
5 which has been produced from alpha-alanine (FIGS. 1 and 3).

Pathways of 3-HP and its Derivatives

As shown in FIG. 1, beta-alanine can be converted into beta-alanyl-CoA through the use of a polypeptide having CoA transferase activity (EC 2.8.3.1) or CoA synthase activity (E.C. 6.2.1.-).

10 Beta-alanine can be produced from alpha-alanine by endogenous polypeptides in a host cell which converts alpha-alanine to beta-alanine, and/or by using a cell transformed with recombinant alanine 2,3-aminomutase, such as a sequence including SEQ ID NO: 20 and/or 29, or fragments, variants, or fusions thereof that retain alanine 2,3-aminomutase activity. Beta-alanyl-CoA can then be converted into acrylyl-CoA through the use of a polypeptide having beta-alanyl-CoA ammonia lyase activity
15 (EC4.3.1.6). Acrylyl-CoA can then be converted into 3-hydroxypropionyl-CoA (3-HP-CoA) through the use of a polypeptide having 3-HP-CoA dehydratase activity (EC 4.2.1.-). 3-HP-CoA can then be converted into 3-HP through several enzymes, including, but not limited to: a polypeptide having CoA transferase activity (EC 2.8.3.1), a polypeptide having 3-hydroxypropionyl-CoA hydrolase activity (EC 3.1.2.-), and a polypeptide having 3-hydroxyisobutryl-CoA hydrolase activity (EC
20 3.1.2.4) can be used to convert 3-HP-CoA into 3-HP.

As shown in FIG. 1, 3-HP can be made from beta-alanine by use of a polypeptide having 4-aminobutyrate and/or beta-alanine-2-oxoglutarate aminotransferase activity which generates malonic semialdehyde from beta-alanine. The malonic semialdehyde can be converted into 3-HP with a polypeptide having 3-HP dehydrogenase activity (EC 1.1.1.59) or a polypeptide having 3-hydroxyisobutyrate dehydrogenase activity (EC 1.1.1.31).

Derivatives of 3-HP can be made from beta-alanine as shown in FIG. 1. The resulting 3-HP-CoA can be converted into polymerized 3-HP by a polypeptide having poly hydroxyacid synthase activity (EC 2.3.1.-). Alternatively or in addition, 3-HP-CoA can be converted into 1,3-propanediol by polypeptides having oxidoreductase activity or reductase activity.

30 The resulting acrylyl-CoA can be converted into polymerized acrylate by a polypeptide having poly hydroxyacid synthase activity (EC 2.3.1.-). Alternatively or in addition, acrylyl-CoA can be converted into acrylate by a polypeptide having CoA transferase activity and/or CoA hydrolase activity; and the resulting acrylate can be converted into an ester of acrylate by a polypeptide having lipase or esterase activity.

35 The resulting 3-HP can be converted into an ester of 3-HP by a polypeptide having lipase or esterase activity (EC 3.1.1.-). Alternatively or in addition, 1,3-propanediol can be created from 3-HP, by a combination of a polypeptide having aldehyde dehydrogenase activity and a polypeptide having alcohol dehydrogenase activity.

Pathways of Pantothenate and its Derivatives

As shown in FIG. 3, pantothenate can be made from beta-alanine by a peptide having alpha-ketopantoate hydroxymethyltransferase (E.C. 2.1.2.11), alpha-ketopantoate reductase (E.C. 1.1.1.169), and pantothenate synthase (E.C. 6.3.2.1) activities, which converts beta-alanine to

5 pantothenate.

Derivatives of pantothenate can be made from beta-alanine as follows. The resulting pantothenate can be converted into CoA by polypeptides having pantothenate kinase (E.C. 2.7.1.33), 4'-phosphopantethenoyl-1-cysteine synthetase (E.C. 6.3.2.5), 4'-phosphopantethenoylcysteine decarboxylase (E.C. 4.1.1.36), ATP:4'-phosphopantetheine adenyltransferase (E.C. 2.7.7.3), and

10 dephospho-CoA kinase (E.C. 2.7.1.24) activities.

Enzymes

Polypeptides having lysine 2,3-aminomutase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, but not limited to: *Clostridium subterminale*, *E. coli*, *B. subtilis*, *Deinococcus radiodurans*, *Porphyromonas gingivalis*, *Aquifex aeolicus*, or *Haemophilus influenza*. For example, amino acid sequences having lysine 2,3-aminomutase activity are shown in SEQ ID NO: 31 for *B. subtilis* and in SEQ ID NO: 28 for *P. gingivalis*.

In another example, a nucleic acid that encodes a polypeptide having alanine 2,3-aminomutase activity is shown in SEQ ID NO: 20 for *B. subtilis* (the corresponding amino acid sequence is shown in SEQ ID NO: 21), and in SEQ ID NO: 29 for *P. gingivalis* (the corresponding amino acid sequence is shown in SEQ ID NO: 30). In addition, other polypeptides having alanine 2,3-aminomutase activity as well as nucleic acids encoding such polypeptides, can be obtained using the methods described herein. For example, alanine 2,3-aminomutase variants can be used to encode a polypeptide having alanine 2,3-aminomutase activity as described above.

Polypeptides having CoA transferase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, but not limited to, *Megasphaera elsdenii*, *Clostridium propionicum*, *Clostridium kluyveri*, and *E. coli*. For example, nucleic acid that encodes a polypeptide having CoA transferase activity is shown in SEQ ID NO: 24 for *M. elsdenii*.

30 In addition, polypeptides having CoA transferase activity (SEQ ID NO: 25) as well as nucleic acid encoding such polypeptides (SEQ ID NO: 24) can be obtained as described herein. For example, CoA transferase variants can be used to encode a polypeptide having CoA transferase activity. For example, the following variations can be made to the CoA transferase nucleic acid sequence (SEQ ID NO: 24): the "a" at position 49 can be substituted with an "c"; the "a" at position 590 can be substituted with a "atgg"; an "aaac" can be inserted before the "g" at position 393; or the "gaa" at position 736 can be deleted. It will be appreciated that the sequences set forth in the sequence listing can contain any number of variations as well as any combination of types of variations, as long as the peptide retains CoA transferase activity. In addition, the following variations can be made to the

CoA transferase amino acid sequence shown in SEQ ID NO: 25: the "k" at position 17 of can be substituted with a "p" or "h"; and the "v" at position 125 can be substituted with an "i" or "f."

5 Polypeptides having beta-alanyl-CoA ammonia lyase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *C. propionicum*. For example, nucleic acid encoding a polypeptide complex having beta-alanyl-CoA ammonia lyase activity can be obtained from *C. propionicum* as described in EXAMPLE 10. The nucleic acid encoding a beta-alanyl-CoA ammonia lyase can contain a sequence as set forth in SEQ ID NO: 22. In addition, polypeptides having beta-alanyl-CoA ammonia lyase activity (SEQ ID NO: 23) as well as nucleic acid encoding such polypeptides (SEQ ID NO: 22) can be obtained as 10 described herein. For example, the variations to the beta-alanyl-CoA ammonia lyase sequence shown in SEQ ID NO: 22 can be used to encode a polypeptide having beta-alanyl-CoA ammonia lyase activity.

15 Polypeptides having 3-hydroxypropionyl-CoA dehydratase activity (also referred to as acrylyl-CoA hydratase activity) as well as nucleic acid encoding such polypeptides can be obtained from various species including, but not limited to, *Chloroflexus aurantiacus*, *Candida rugosa*, *Rhodospirillum rubrum*, and *Rhodobacter capsulatus*. For example, a nucleic acid that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity is disclosed in WO 02/42418.

20 Polypeptides having glutamate dehydrogenase activity as well as nucleic acid encoding such polypeptides can be obtained from various species.

25 Polypeptides having 3-hydroxypropionyl-CoA or 3-hydroxyisobutyryl-CoA hydrolase activity, as well as nucleic acid encoding such polypeptides, can be obtained from various species including, without limitation, *Pseudomonas fluorescens*, *Rattus rattus*, and *Homo sapiens*. For example, nucleic acid that encodes a polypeptide having 3-hydroxyisobutyryl-CoA hydrolase activity can be obtained from *H. sapiens* and can have a sequence as set forth in GenBank accession number U66669.

Polypeptides having 4-aminobutyrate and/or beta-alanine-2-oxoglutarate aminotransferase activity, 3-HP dehydrogenase activity, and 3-hydroxyisobutyrate dehydrogenase activity, as well as nucleic acid encoding such polypeptides can be obtained from various species.

30 Polypeptides having poly hydroxyacid synthase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Rhodobacter sphaeroides*, *Comamonas acidivorans*, *Ralstonia eutropha*, and *Pseudomonas oleovorans*. For example, nucleic acid that encodes a polypeptide having poly hydroxyacid synthase activity can be obtained from *R. sphaeroides* and can have a sequence as set forth in GenBank accession number X97200. Additional information about poly hydroxyacid synthase can be found in Song *et al.* 35 (*Biomacromolecules* 1:433-9, 2000).

Polypeptides having acetylating aldehyde:NAD(+) oxidoreductase activity (EC 1.2.1.10) as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *E. coli*. For example, nucleic acid that encodes a polypeptide having acetylating

aldehyde dehydrogenase activity can be obtained from *E. coli* and can have a sequence as set forth in GenBank accession number Y09555.

5 Aldehyde:NAD(+) oxidoreductase activity and alcohol:NAD(+) oxidoreductase activities can be carried out by two different polypeptides as described above, or carried out by a single polypeptide, such as a multi-functional aldehyde-alcohol dehydrogenase (EC 1.2.1.10) from *E. coli* (Goodlove *et al.* *Gene* 85:209-14, 1989; GenBank Accession No. M33504).

10 Polypeptides having aldehyde dehydrogenase (NAD(P)+) (EC 1.2.1.-) activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *S. cerevisiae*. For example, nucleic acid that encodes a polypeptide having aldehyde dehydrogenase activity can be obtained from *S. cerevisiae* and can have a sequence as set forth in GenBank Accession No. Z75282 (Tessier *et al.* *FEMS Microbiol. Lett.* 164:29-34, 1998).

15 Polypeptides having alcohol dehydrogenase activity (EC 1.1.1.1) as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Z. mobilis*. For example, nucleic acid that encodes a polypeptide having alcohol dehydrogenase activity can be obtained from *Z. mobilis* and can have a sequence as set forth in GenBank accession No. M32100.

20 Polypeptides having lipase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Candida rugosa*, *Candida tropicalis*, and *Candida albicans*. For example, nucleic acid that encodes a polypeptide having lipase activity can be obtained from *C. rugosa* and can have a sequence as set forth in GenBank accession number A81171.

25 Polypeptides having alpha-ketopantoate hydroxymethyltransferase and pantothenate synthase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *E. coli*. For example, nucleic acids that encodes polypeptides having alpha-ketopantoate hydroxymethyltransferase and pantothenate synthase activity can be obtained from *E. coli* and can have a sequence as set forth in GenBank accession number L17086.

30 Polypeptides having alpha-ketopantoate reductase, pantothenate kinase, 4'-phosphopantethenoyl-1-cysteine synthetase, 4'-phosphopantethenoylcysteine decarboxylase, ATP:4'-phosphopantetheine adenyltransferase, and dephospho-CoA kinase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *E. coli*. For example, nucleic acids that encodes polypeptides having alpha-ketopantoate reductase pantothenate kinase, 4'-phosphopantethenoyl-1-cysteine synthetase, 4'-phosphopantethenoylcysteine decarboxylase, ATP:4'-phosphopantetheine adenyltransferase, and dephospho-CoA kinase activity can be obtained from *E. coli* and can have a sequence as set forth in GenBank accession number NC000913.

35 The term "polypeptide having enzymatic activity" refers to any polypeptide that catalyzes a chemical reaction of other substances without itself being destroyed or altered upon completion of the reaction. Typically, a polypeptide having enzymatic activity catalyzes the formation of one or more

products from one or more substrates. Such polypeptides can have any type of enzymatic activity including, without limitation, the enzymatic activity or enzymatic activities associated with enzymes such as alanine 2,3-aminomutase, dehydratases/hydratases, 3-hydroxypropionyl-CoA dehydratases/hydratases, alanine dehydrogenase, CoA transferases, 3-hydroxypropionyl-CoA hydrolases, 3-hydroxyisobutryl-CoA hydrolases, CoA hydrolases, poly hydroxyacid synthases, beta-alanine ammonia lyases, 4-aminobutyrate or beta-alanine-2-oxoglutarate aminotransferases, 3-HP dehydrogenases, 3-hydroxyisobutyrate dehydrogenases, glutamate dehydrogenases, lipases, esterases, acetylating aldehyde:NAD(+) oxidoreductases, alcohol:NAD(+) oxidoreductases, aldehyde dehydrogenases, alcohol dehydrogenases hydroxymethyltransferases, reductases, synthases, kinases, synthetases, decarboxylases, alpha-ketopantoate hydroxymethyltransferases, alpha-ketopantoate reductases, pantothenate synthases, pantothenate kinases, 4'-phosphopantethenoyl-1-cysteine synthetase, 4'-phosphopantethenoylcysteine decarboxylases, ATP:4'-phosphopantetheine adenyltransferases, dephospho-CoA kinases, acetylating aldehyde:NAD(+) oxidoreductases, alcohol:NAD(+) oxidoreductases, aldehyde dehydrogenases (NAD(P)+), alcohol dehydrogenases and adenyltransferases.

Methods of Making 3-HP, Pantothenate, and Derivatives Thereof

Each step provided in the pathways depicted in FIGS. 1 and 3 can be performed within a cell (*in vivo*) or outside a cell (*in vitro*, e.g., in a container or column). Additionally, the organic compound products can be generated through a combination of *in vivo* synthesis and *in vitro* synthesis. Moreover, the *in vitro* synthesis step, or steps, can be via chemical reaction or enzymatic reaction.

For example, a cell or microorganism provided herein can be used to perform the steps provided in FIGS. 1 and 3, or an extract containing polypeptides having the indicated enzymatic activities can be used to perform the steps provided in FIGS. 1 and 3. In addition, chemical treatments can be used to perform the conversions provided in FIGS. 1 and 3. For example, acrylyl-CoA can be converted into acrylate by hydrolysis. Other chemical treatments include, without limitation, trans esterification to convert acrylate into an acrylate ester.

30 Expression of Polypeptides

The polypeptides described herein, such as the enzymes listed in FIG. 1, can be produced individually in a host cell or in combination in a host cell. Moreover, the polypeptides having a particular enzymatic activity can be a polypeptide that is either naturally-occurring or non-naturally-occurring. A naturally-occurring polypeptide is any polypeptide having an amino acid sequence as found in nature, including wild-type and polymorphic polypeptides. Naturally-occurring polypeptides can be obtained from any species including, but not limited to, animal (e.g., mammalian), plant, fungal, and bacterial species. A non-naturally-occurring polypeptide is any polypeptide having an amino acid sequence that is not found in nature. Thus, a non-naturally-

occurring polypeptide can be a mutated version of a naturally-occurring polypeptide, or an engineered polypeptide. For example, a non-naturally-occurring polypeptide having alanine 2,3-aminomutase activity can be a mutated version of a naturally-occurring polypeptide having lysine 2,3-aminomutase activity that has at least some alanine 2,3-aminomutase activity (such as SEQ ID NO: 21 and/or 30). A polypeptide can be mutated by, for example, sequence additions, deletions, substitutions, or combinations thereof.

Genetically modified cells are disclosed which can be used to perform one or more steps of the steps in the pathways described herein or the genetically modified cells can be used to produce the disclosed polypeptides for subsequent use *in vitro*. For example, an individual microorganism 10 can contain exogenous nucleic acid(s) encoding each of the polypeptides necessary to perform the steps depicted in FIGS. 1 and 3. Such cells can contain any number of exogenous nucleic acid molecules. For example, a particular cell can contain one, two, three, or four different exogenous nucleic acid molecules with each one encoding the polypeptide(s) necessary to convert pyruvate into 3-HP as shown in FIG. 1, or a particular cell can endogenously produce polypeptides necessary to 15 convert pyruvate into acrylyl-CoA while containing exogenous nucleic acid that encodes polypeptides necessary to convert acrylyl-CoA into 3-HP.

In addition, a single exogenous nucleic acid molecule can encode one, or more than one, polypeptide. For example, a single exogenous nucleic acid molecule can contain sequences that encode two, three, or even four different polypeptides. Further, the cells described herein can contain 20 a single copy, or multiple copies (e.g., about 5, 10, 20, 35, 50, 75, 100 or 150 copies), of a particular exogenous nucleic acid molecule, such as a particular enzyme. The cells described herein can contain more than one particular exogenous nucleic acid. For example, a particular cell can contain about 50 copies of exogenous nucleic acid molecule X as well as about 75 copies of exogenous nucleic acid molecule Y.

25 In another example, a cell can contain an exogenous nucleic acid molecule that encodes a polypeptide having alanine 2,3-aminomutase activity, for example SEQ ID NO: 20 and/or 29 (or variants, fragments, or fusions thereof that retain alanine 2,3-aminomutase activity). Such cells can have any detectable level of alanine 2,3-aminomutase activity, including activity detected by the production of metabolites of beta-alanine, such as pantothenate. For example, a cell containing an 30 exogenous nucleic acid molecule that encodes a polypeptide having alanine 2,3-aminomutase activity can have alanine 2,3-aminomutase activity with a specific activity greater than about 1 μ g beta-alanine formed per gram dry cell weight per hour (e.g., greater than about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 250, 300, 350, 400, 500, or more μ g beta-alanine formed per gram dry cell weight per hour). Alternatively, a cell can have alanine 2,3-aminomutase activity such that a cell 35 extract from 1×10^6 cells has a specific activity greater than about 1 ng beta-alanine formed per mg total protein per minute (e.g., greater than about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 250, 300, 350, 400, 500, or more ng beta-alanine formed per mg total protein per minute).

A nucleic acid molecule encoding a polypeptide having enzymatic activity can be identified and obtained using any method such as those described herein. For example, nucleic acid molecules that encode a polypeptide having enzymatic activity can be identified and obtained using common molecular cloning or chemical nucleic acid synthesis procedures and techniques, including PCR. In 5 addition, standard nucleic acid sequencing techniques and software programs that translate nucleic acid sequences into amino acid sequences based on the genetic code can be used to determine whether or not a particular nucleic acid has any sequence homology with known enzymatic polypeptides. Sequence alignment software such as MEGALIGN (DNASTAR, Madison, WI, 1997) can be used to compare various sequences.

10 In addition, nucleic acid molecules encoding known enzymatic polypeptides can be mutated using common molecular cloning techniques (e.g., site-directed mutagenesis). Possible mutations include, without limitation, deletions, insertions, and base substitutions, as well as combinations of deletions, insertions, and base substitutions. Further, nucleic acid and amino acid databases (e.g., GenBank) can be used to identify a nucleic acid sequence that encodes a polypeptide having enzymatic activity. Briefly, any amino acid sequence having some homology to a polypeptide having enzymatic activity, or any nucleic acid sequence having some homology to a sequence encoding a polypeptide having enzymatic activity can be used as a query to search GenBank. The identified 15 polypeptides then can be analyzed to determine whether or not they exhibit enzymatic activity.

20 In addition, nucleic acid hybridization techniques can be used to identify and obtain a nucleic acid molecule that encodes a polypeptide having enzymatic activity. Briefly, any nucleic acid molecule that encodes a known enzymatic polypeptide, or fragment thereof, can be used as a probe to identify similar nucleic acid molecules by hybridization under conditions of moderate to high stringency. Such similar nucleic acid molecules then can be isolated, sequenced, and analyzed to determine whether the encoded polypeptide has enzymatic activity.

25 Expression cloning techniques also can be used to identify and obtain a nucleic acid molecule that encodes a polypeptide having enzymatic activity. For example, a substrate known to interact with a particular enzymatic polypeptide can be used to screen a phage display library containing that enzymatic polypeptide. Phage display libraries can be generated as described (Burritt *et al.*, *Anal. Biochem.* 238:1-13, 1990), or can be obtained from commercial suppliers such as 30 Novagen (Madison, WI).

35 Further, polypeptide sequencing techniques can be used to identify and obtain a nucleic acid molecule that encodes a polypeptide having enzymatic activity. For example, a purified polypeptide can be separated by gel electrophoresis, and its amino acid sequence determined by, for example, amino acid microsequencing techniques. Once determined, the amino acid sequence can be used to design degenerate oligonucleotide primers. Degenerate oligonucleotide primers can be used to obtain the nucleic acid encoding the polypeptide by PCR. Once obtained, the nucleic acid can be sequenced, cloned into an appropriate expression vector, and introduced into a microorganism.

Any method can be used to introduce an exogenous nucleic acid molecule into a cell. For example, heat shock, lipofection, electroporation, conjugation, fusion of protoplasts, and biolistic delivery are common methods for introducing nucleic acid into bacteria and yeast cells. (See, e.g., Ito *et al.*, *J. Bacteriol.* 153:163-8, 1983; Durrens *et al.*, *Curr. Genet.* 18:7-12, 1990; Sambrook *et al.*, 5 Molecular cloning: A laboratory manual, Cold Spring Harbour Laboratory Press, New York, USA, second edition, 1989; and Becker and Guarente, *Methods in Enzymology* 194:182-7, 1991). Other methods for expressing an amino acid sequence from an exogenous nucleic acid molecule include, but are not limited to, constructing a nucleic acid such that a regulatory element promotes the expression of a nucleic acid sequence that encodes a polypeptide. Typically, regulatory elements are 10 DNA sequences that regulate the expression of other DNA sequences at the level of transcription. Thus, regulatory elements include, without limitation, promoters, enhancers, and the like. Any type of promoter can be used to express an amino acid sequence from an exogenous nucleic acid molecule. Examples of promoters include, without limitation, constitutive promoters, tissue-specific promoters, and promoters responsive or unresponsive to a particular stimulus (e.g., light, oxygen, 15 chemical concentration). Methods for transferring nucleic acids into mammalian cells are also known, such as using viral vectors.

An exogenous nucleic acid molecule contained within a particular cell of the disclosure can be maintained within that cell in any form. For example, exogenous nucleic acid molecules can be integrated into the genome of the cell or maintained in an episomal state. That is, a cell can be a 20 stable or transient transformant. A microorganism can contain single or multiple copies (e.g., about 5, 10, 20, 35, 50, 75, 100 or 150 copies), of a particular exogenous nucleic acid molecule, such as a nucleic acid encoding an enzyme.

Production of Organic Acids and Related Products via Host Cells

25 The nucleic acid and amino acid sequences provided herein can be used with cells to produce beta-alanine, pantothenate and 3-HP, as well as derivatives thereof such as CoA, and organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3-HP, and polymerized 3-HP. Such cells can be from any species, such as those listed within the taxonomy web pages at the National Institutes of Health. The cells can be eukaryotic or prokaryotic. 30 For example, genetically modified cells can be mammalian cells (e.g., human, murine, and bovine cells), plant cells (e.g., corn, wheat, rice, and soybean cells), fungal cells (e.g., *Aspergillus* and *Rhizopus* cells), yeast cells, or bacterial cells (e.g., *Lactobacillus*, *Lactococcus*, *Bacillus*, *Escherichia*, and *Clostridium* cells). In one example, a cell is a microorganism. The term "microorganism" refers to any microscopic organism including, but not limited to, bacteria, algae, fungi, and protozoa. Thus, 35 *E. coli*, *B. subtilis*, *B. licheniformis*, *S. cerevisiae*, *Kluveromyces lactis*, *Candida blankii*, *Candida rugosa*, and *Pichia pastoris* are microorganisms and can be used as described herein. In another example, the cell is part of a larger organism, such as a plant, such as a transgenic plant. Examples of plants that can be used to make 3-HP, pantothenate, or other organic compounds from beta-alanine

include, but are not limited to, genetically engineered plant crops such as corn, rice, wheat, and soybean.

5 In one example, a cell is genetically modified such that a particular organic compound is produced. In one embodiment, cells make 3-HP and/or pantothenate from beta-alanine, such as the pathways shown in FIGS. 1 and 3. In another embodiment, the cells make derivatives of 3-HP and/or pantothenate, such as CoA, and organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3-HP, and polymerized 3-HP.

10 In one example, cells that are genetically modified to synthesize a particular organic compound contain one or more exogenous nucleic acid molecules that encode polypeptides having specific enzymatic activities. For example, a microorganism can contain exogenous nucleic acid that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity. In this case, acrylyl-CoA can be converted into 3-hydroxypropionic acid-CoA which can lead to the production of 3-HP. A cell can be given an exogenous nucleic acid molecule that encodes a polypeptide having an enzymatic activity that catalyzes the production of a compound not normally produced by that cell.

15 Alternatively, a cell can be given an exogenous nucleic acid molecule that encodes a polypeptide having an enzymatic activity that catalyzes the production of a compound that is normally produced by that cell. In this case, the genetically modified cell can produce more of the compound, or can produce the compound more efficiently, than a similar cell not having the genetic modification.

20 In another example, a cell containing an exogenous nucleic acid molecule that encodes a polypeptide having enzymatic activity that leads to the formation of 3-HP, pantothenate, and/or derivatives thereof, is disclosed. The produced product(s) can be secreted from the cell, eliminating the need to disrupt cell membranes to retrieve the organic compound. In one example, the cell produces 3-HP, pantothenate, and/or derivatives thereof, with the concentration of the product(s) being at least about 100 mg per L (e.g., at least about 1 g/L, 5 g/L, 10 g/L, 25 g/L, 50 g/L, 75 g/L, 80 g/L, 90 g/L, 100 g/L, or 120 g/L). When determining the yield of a compound such as 3-HP, pantothenate, and/or derivatives thereof for a particular cell, any method can be used. See, e.g., *Applied Environmental Microbiology* 59(12):4261-5 (1993). A cell within the scope of the disclosure can utilize a variety of carbon sources.

25 A cell can contain one or more exogenous nucleic acid molecules that encodes a polypeptide(s) having enzymatic activity that leads to the formation of 3-HP, pantothenate, and/or derivatives thereof, such as CoA, 1,3-propanediol, acrylic acid, poly-acrylate, acrylate-esters, 3-HP-esters, and polymers and copolymers containing 3-HP. Methods of identifying cells that contain exogenous nucleic acid(s) are well known. Such methods include, without limitation, PCR and nucleic acid hybridization techniques such as Northern and Southern analysis (see hybridization described herein). In some cases, immunohisto-chemical and biochemical techniques can be used to determine if a cell contains particular nucleic acid(s) by detecting the expression of the polypeptide(s) encoded by that particular nucleic acid molecule(s). For example, an antibody having specificity for a polypeptide can be used to determine whether or not a particular cell contains nucleic acid encoding

that polypeptide. Further, biochemical techniques can be used to determine if a cell contains a particular nucleic acid molecule encoding a polypeptide having enzymatic activity by detecting an organic product produced as a result of the expression of the polypeptide having enzymatic activity. For example, detection of 3-HP after introduction of exogenous nucleic acid that encodes a 5 polypeptide having 3-hydroxypropionyl-CoA dehydratase activity into a cell that does not normally express such a polypeptide can indicate that the cell not only contains the introduced exogenous nucleic acid molecule but also expresses the encoded polypeptide from that introduced exogenous nucleic acid molecule. Methods for detecting specific enzymatic activities or the presence of particular organic products are well known, for example, the presence of an organic compound such 10 as 3-HP can be determined as described in Sullivan and Clarke (*J. Assoc. Offic. Agr. Chemists*, 38:514-8, 1955).

Cells with Reduced Polypeptide Activity

Genetically modified cells having reduced polypeptide activity are disclosed. The term 15 "reduced" or "decreased" as used herein with respect to a cell and a particular polypeptide's activity refers to a lower level of activity than that measured in a comparable cell of the same species. For example, a particular microorganism lacking enzymatic activity X has reduced enzymatic activity X if a comparable microorganism has at least some enzymatic activity X.

A cell can have the activity of any type of polypeptide reduced including, without limitation, 20 enzymes, transcription factors, transporters, receptors, signal molecules, and the like. For example, a cell can contain an exogenous nucleic acid molecule that disrupts a regulatory and/or coding sequence of a polypeptide having *panD* activity. Disrupting *panD* can prevent a cell from making beta-alanine.

Reduced polypeptide activities can be the result of lower polypeptide concentration, lower 25 specific activity of a polypeptide, or combinations thereof. Many different methods can be used to make a cell having reduced polypeptide activity. For example, a cell can be engineered to have a disrupted regulatory sequence or polypeptide-encoding sequence using common mutagenesis or knock-out technology. (*Methods in Yeast Genetics* (1997 edition), Adams, Gottschling, Kaiser, and Sterns, Cold Spring Harbor Press, 1998; Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97: 6640-5, 2000). Alternatively, antisense technology can be used to reduce the activity of a particular 30 polypeptide. For example, a cell can be engineered to contain a cDNA that encodes an antisense molecule that prevents a polypeptide from being translated. The term "antisense molecule" encompasses any nucleic acid molecule or nucleic acid analog (e.g., peptide nucleic acids) that contains a sequence that corresponds to the coding strand of an endogenous polypeptide. An 35 antisense molecule also can have flanking sequences (e.g., regulatory sequences). Thus, antisense molecules can be ribozymes or antisense oligonucleotides. A ribozyme can have any general structure including, without limitation, hairpin, hammerhead, or axhead structures, provided the

molecule cleaves RNA. Further, gene silencing can be used to reduce the activity of a particular polypeptide.

A cell having reduced activity of a polypeptide can be identified using any method. For example, enzyme activity assays such as those described herein can be used to identify cells having a 5 reduced enzyme activity.

Production of Organic Acids and Related Products via In Vitro Techniques

Purified polypeptides having enzymatic activity can be used alone or in combination with 10 cells to produce pantothenate, 3-HP, and/or derivatives thereof such as CoA, and organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3-HP, and polymerized 3-HP. For example, a preparation including a substantially pure polypeptide having 3-hydroxypropionyl-CoA dehydratase activity can be used to catalyze the formation of 3-HP-CoA, a precursor to 3-HP.

Further, cell-free extracts containing a polypeptide having enzymatic activity can be used 15 alone or in combination with purified polypeptides and/or cells to produce pantothenate, 3-HP, and/or deviates thereof. For example, a cell-free extract which includes a polypeptide having CoA transferase activity can be used to form beta-alanyl-CoA from beta-alanine, while a microorganism containing polypeptides which have the enzymatic activities necessary to catalyze the reactions needed to form 3-HP from beta-alanyl-CoA can be used to produce 3-HP. In another example, a cell-free extract which includes alpha-ketopantoate hydroxymethyltransferase (E.C. 2.1.2.11), alpha-ketopantoate reductase (E.C. 1.1.1.169), and pantothenate synthase (E.C. 6.3.2.1) can be used to form pantothenate from beta-alanine. Any method can be used to produce a cell-free extract. For 20 example, osmotic shock, sonication, and/or a repeated freeze-thaw cycle followed by filtration and/or centrifugation can be used to produce a cell-free extract from intact cells.

25 A cell, purified polypeptide, and/or cell-free extract can be used to produce 3-HP that is, in turn, treated chemically to produce another compound. For example, a microorganism can be used to produce 3-HP, while a chemical process is used to modify 3-HP into a derivative such as polymerized 3-HP or an ester of 3-HP. Likewise, a chemical process can be used to produce a particular compound that is, in turn, converted into 3-HP or other organic compound (e.g., 1,3-propanediol, 30 acrylic acid, polymerized acrylate, esters of acrylate, esters of 3-HP, and polymerized 3-HP) using a cell, substantially pure polypeptide, and/or cell-free extract described herein. For example, a chemical process can be used to produce acrylyl-CoA, while a microorganism can be used convert acrylyl-CoA into 3-HP.

35 Similarly, a cell, purified polypeptide, and/or cell-free extract can be used to produce pantothenate that is, in turn, treated chemically to produce another compound. For example, a microorganism can be used to produce pantothenate, while a chemical process is used to modify pantothenate into a derivative such as CoA. Likewise, a chemical process can be used to produce a particular compound that is, in turn, converted into pantothenate or other compound (e.g., CoA) using

a cell, substantially pure polypeptide, and/or cell-free extract described herein. For example, a chemical process can be used to produce pantothenate, while a microorganism can be used convert pantothenic acid into CoA.

5 *Fermentation of Cells to Produce Organic Acids*

A method for producing pantothenate, 3-HP, and/or derivatives thereof by culturing a production cells, such as a microorganism, in culture medium such that pantothenate, 3-HP, and/or derivatives thereof, is produced, is disclosed. In general, the culture media and/or culture conditions can be such that the microorganisms grow to an adequate density and produce the product efficiently.

10 For large-scale production processes, any method can be used such as those described elsewhere (Manual of Industrial Microbiology and Biotechnology, 2nd Edition, Editors: Demain and Davies, ASM Press; and Principles of Fermentation Technology, Stanbury and Whitaker, Pergamon).

Briefly, a large tank (e.g., a 100 gallon, 200 gallon, 500 gallon, or more tank) containing appropriate culture medium with, for example, a glucose carbon source is inoculated with a particular microorganism. After inoculation, the microorganisms are incubated to allow biomass to be produced. Once a desired biomass is reached, the broth containing the microorganisms can be transferred to a second tank. This second tank can be any size. For example, the second tank can be larger, smaller, or the same size as the first tank. Typically, the second tank is larger than the first such that additional culture medium can be added to the broth from the first tank. In addition, the culture medium within this second tank can be the same as, or different from, that used in the first tank. For example, the first tank can contain medium with xylose, while the second tank contains medium with glucose.

Once transferred, the microorganisms can be incubated to allow for the production of pantothenate, 3-HP, and/or derivatives thereof. Once produced, any method can be used to isolate the formed product. For example, common separation techniques can be used to remove the biomass from the broth, and common isolation procedures (e.g., extraction, distillation, and ion-exchange procedures) can be used to obtain the pantothenate, 3-HP, and/or derivatives thereof from the microorganism-free broth. Alternatively, the product can be isolated while it is being produced, or it can be isolated from the broth after the product production phase has been terminated.

30

Products Created From the Disclosed Biosynthetic Routes

The compounds produced from any of the steps provided in FIGS. 1 and 3 can be chemically converted into other organic compounds. For example, 3-HP can be hydrogenated to form 1,3-propanediol, a valuable polyester monomer. Hydrogenating an organic acid such as 3-HP can be performed using any method such as those used to hydrogenate succinic acid and/or lactic acid. For example, 3-HP can be hydrogenated using a metal catalyst. In another example, 3-HP can be dehydrated to form acrylic acid. Any method can be used to perform a dehydration reaction. For example, 3-HP can be heated in the presence of a catalyst (e.g., a metal or mineral acid catalyst) to

form acrylic acid. 1,3-propanediol also can be created using polypeptides having oxidoreductase activity (e.g., enzymes in the 1.1.1.- class of enzymes) *in vitro* or *in vivo*.

In another example, pantothenate can be used to form coenzyme A. Polypeptides having pantothenate kinase (E.C. 2.7.1.33), 4'-phosphopantethenyl-1-cysteine synthetase (E.C. 6.3.2.5), 4'-phosphopantethenoylcysteine decarboxylase (E.C. 4.1.1.36), ATP:4'-phosphopantetheine adenyltransferase (E.C. 2.7.7.3), and dephospho-CoA kinase (E.C. 2.7.1.24) activities can be used to produce coenzyme A.

Production of 1,3-propanediol

10 Methods of producing 1,3-propanediol, and cells for such production, are disclosed. 1,3-propanediol can be generated from either 3-HP-CoA or 3-HP. Cells or microorganisms producing 3-HP-CoA or 3-HP can be engineered to make 1,3-propanediol by cloning genes which encode for enzymes having oxidoreductase/dehydrogenase type activity.

15 For example, 3-HP-CoA can be converted to 1,3-propanediol in the presence of an enzyme having acetylating aldehyde:NAD(+) oxidoreductase and alcohol:NAD(+) oxidoreductase activities. Such conversion can be performed *in vivo*, *in vitro*, or a combination thereof. These activities can be carried out by a single polypeptide or by two different polypeptides. Single enzymes include the multi-functional aldehyde-alcohol dehydrogenase (EC 1.2.1.10) from *E. coli* (Goodlove *et al.* *Gene* 85:209-14, 1989; GenBank Accession No. M33504). Enzymes having a singular activity of acetylating aldehyde:NAD(+) oxidoreductase (EC 1.2.1.10) or alcohol:NAD(+) oxidoreductase (EC 1.1.1.1) have been described. Genes encoding for acylating aldehyde dehydrogenase from *E. coli* (GenBank Accession No. Y09555) and alcohol dehydrogenase from *Z. mobilis* (GenBank Accession No. M32100) have been isolated and sequenced. The genes encoding for these enzymes can be cloned into a 3-HP-CoA producing organism or cell by well-known molecular biology techniques.

20 Expression of these enzymes in 3-HP-CoA producing organisms or cells will impart it the ability to convert 3-HP-CoA to 1,3-propanediol. The substrate specificity of these enzymes for 3-HP-CoA can be changed or improved using well-known techniques such as error prone PCR or mutator *E. coli* strains.

25

30 Conversion of 3-HP to 1,3-propanediol can be achieved by contacting 3-HP with enzymes having aldehyde dehydrogenase (NAD(P)+) (EC 1.2.1.-) and alcohol dehydrogenase (EC 1.1.1.1) activity. Such conversion can be performed *in vivo*, *in vitro*, or a combination thereof. For example, cloning and expressing these genes in a 3-HP producing microorganism or cell will impart the ability of the cell or organism to convert 3-HP to 1,3-propanediol. The substrate specificity of these enzymes for 3-HP-CoA can be changed or improved using well-known techniques as described above.

35 The formation of 1,3-propanediol during fermentation or in an *in vitro* assay can be analyzed using a High Performance Liquid Chromatography (HPLC). The chromatographic separation can be achieved by using a Bio-Rad 87H ion-exchange column. A mobile phase of 0.01N sulfuric acid is

passed at a flow rate of 0.6 ml/min and the column maintained at a temperature of 45-65°C. The presence of 1,3-propanediol in the sample can be detected using a refractive index detector (Skraly *et al.*, *Appl. Environ. Microbiol.* 64:98-105, 1998).

5

EXAMPLE 1

Cloning a *Bacillus subtilis* lysine 2,3-aminomutase (*KAM* gene)

To identify an alanine 2,3-aminomutase that produces beta-alanine for alanine, enzymes which carry out similar reactions, but which do not accept alanine or beta-alanine as substrates, were 10 randomly mutated and then screened to identify mutant enzymes that have alanine 2,3-aminomutase activity. This example describes cloning lysine 2,3-aminomutase (E.C. 5.4.3.2) from *Bacillus subtilis* (SEQ ID NO: 3 and 31). One skilled in the art will understand that similar methods can be used to clone a lysine 2,3-aminomutase from any desired organism.

15 The *B. subtilis* lysine 2,3-aminomutase was chosen because it was reported to be stable to air, thus permitting selection for activity under both anaerobic and aerobic conditions. In addition, because this enzyme has lower specific activity than the lysine 2,3-aminomutase of *C. subterminale*, deleterious effects to the *E. coli* host by overexpression of active lysine 2,3-aminomutase or alanine 2,3-aminomutase were reduced.

20 To clone the *B. subtilis* *KAM* gene encoding lysine 2,3-aminomutase, the following methods were used. *B. subtilis* ATCC 6051 was obtained from ATCC (American Type Culture Collection (ATCC), Manassas, VA) and chromosomal DNA prepared using the Genomic Tip 20/G (Qiagen, Valencia, CA) following the procedure recommended by the manufacturer. Primers designed to 25 amplify the *KAM* gene by PCR were based on the complete *B. subtilis* genome sequence (GenBank Accession No: NC 000964) and the sequences disclosed in Chen *et al.* (*Biochem. J.* 348:539-49, 2000) and in U.S. Patent No. 6,248,874. The PCR primers:

GCGCGAGGAGGAGTTCATATGAAAAACAAATGGTATAAAC (SEQ ID NO: 1), and CGGGCACCGCTTCGAGGC GGC CGC ACCATTCGCCATG (SEQ ID NO: 2) were used, where the underlined nucleotides are the *Nde*I and *Not*I sites used for cloning the PCR product into plasmids.

30 The PCR reaction (100 µl total volume) contained 0.5 µg *B. subtilis* chromosomal DNA, 0.2 µM each primer (SEQ ID NOS: 1 and 2), 10 µL 10X PfuTurbo reaction buffer (Stratagene, Inc., La Jolla, CA), 0.2 mM each nucleotide triphosphate, and 5 units of PfuTurbo DNA polymerase (Stratagene). The PCR reaction was heated at 95°C for 2 minutes, then subjected to 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 2 minutes, and then held at 72° for an additional 35 10 minutes.

The resulting PCR product was precipitated by the addition of 3 µl Pellet Paint Co-Precipitant (Novagen, Inc., Madison, WI), 100 µl 5M ammonium acetate, and 400 µl ethanol. The resuspended reaction was digested with *Nde*I and *Not*I (New England Biolabs, Inc., Beverly, MA),

purified with the QIAquick PCR Purification Kit (Qiagen), and ligated with the Rapid DNA Ligation Kit (Roche Molecular Biochemicals, Indianapolis, IN) into pET-22b(+) (Novagen) or pPRONde digested with the same enzymes to generate the plasmids pET-KAM1 and pPRO-KAM1, respectively. Plasmid pPRONde is a derivative of pPROLar.A122 (Clontech Laboratories, Inc., Palo Alto, CA) in which an *Nde*I site was constructed at the initiator ATG codon by oligonucleotide-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit from Stratagene. Expression of lysine 2,3-aminomutase in these vectors is driven by the T7 promoter in pET22(b) or a hybrid lac/ara promoter in pPRO-Nde. Ligations were transformed into *E. coli* DH5 α (Life Technologies, Gaithersburg, MD) and clones verified by sequencing. The *B. subtilis* *KAM* gene is shown in SEQ ID NO: 3 (the amino acid sequence is shown in SEQ ID NO: 31), and was mutagenized as described below, to identify mutants having alanine 2,3-aminomutase activity.

EXAMPLE 2

in vitro mutagenesis of a *B. subtilis* *KAM* gene

To introduce mutations into the *B. subtilis* *KAM* gene (SEQ ID NO: 3) *in vitro*, several error-prone PCR methods were used. Similar methods can be used to introduce mutations into any KAM gene encoding a lysine 2,3-aminomutase, such as a *KAM* gene from *Deinococcus radiodurans* (GenBank Accession No: RDR02336), which is 52% identical to the *B. subtilis* *KAM* protein sequence, *Clostridium subterminale* (GenBank Accession No: AF159146), or *P. gingivalis* (Incomplete genome, The Institute for Genomic Research, see EXAMPLE 5).

In one method, a GeneMorph PCR Mutagenesis Kit (Stratagene) was used as follows. Reactions of 50 μ L were set up with 10, 1, 0.1, or 0.01 ng of template pET-KAM1 DNA and 125 ng each of the T7 promoter primer and the T7 terminator primer (sequences as given in the Novagen product catalog) as recommended by the manufacturer, heated at 94°C for 30 seconds, subjected to 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 2 minutes, and then held at 72° for an additional 10 minutes.

The resulting PCR products were precipitated with 3 μ l Pellet Paint Co-Precipitant (Novagen), 50 μ l 5M ammonium acetate, 200 μ l ethanol, resuspended, digested with *Nde*I and *Not*I, and 120 ng of each mutagenic PCR product was ligated to pPRONde digested with the same endonucleases using the Rapid DNA Ligation Kit (Roche Molecular Biochemicals). The ligation mixes were digested with restriction endonuclease *Bam*H1 (New England Biolabs) to linearize residual vector DNA without insert, precipitated with ethanol as described, and transformed into electrocompetent ElectroMax DH10B *E. coli* cells (Invitrogen, Carlsbad, CA). Kanamycin-resistant transformants from each library, containing 15,000-20,000 clones, were scrapped off the selection plates and the mutagenized plasmid libraries prepared using Plasmid Midi Kit (Qiagen).

In a second method, based on the Mn-dITP PCR method of Xu *et al.* (*BioTechniques* 27:1102-8, 1999), an initial round of manganese-induced error-prone PCR was conducted using pET-KAM1 DNA as template and primers homologous to the T7 promoter and T7 terminator regions.

The reaction mixture (50 μ L) contained 1X Taq PCR buffer with 2 mM MgCl₂, 100 ng of DNA, 40 μ M MnCl₂, 0.2 μ M of each primer, 200 μ M of each dNTP, and five units of Taq polymerase (Roche Molecular Biochemicals). The PCR program included of an initial denaturation at 94°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 54°C for 1 minute, and 72°C for 2.25 minutes; and a final extension at 72°C for 7 minutes.

5 Three microliters of PCR product was used as template in a second round of PCR using dITP to enhance mispairing of nucleotides during amplification. The second-round PCR mixture (100 μ L) contained 1X Taq polymerase PCR buffer with 2 mM MgCl₂, 40 μ M dITP, 0.2 μ M of each primer, 200 μ M of each dNTP, and 10 units of Taq DNA polymerase. The PCR program was
10 identical to that for the first round but consisted of 30 cycles. The PCR product was separated on a 1% TAE-agarose gel and purified using a QIAquick Gel Purification procedure (Qiagen). The purified PCR product was digested with the restriction enzymes *Nde*I and *Not*I and ligated into pPRO-Nde vector that had been digested with the same enzymes, gel purified, and dephosphorylated with shrimp alkaline phosphatase (Roche). The ligation reaction was conducted using T4 DNA
15 ligase (New England BioLabs) at 16°C for 16 hours, after which another volume of 1X ligation buffer and ligase was added and the reaction continued for two hours at room temperature.

The ligation reaction was purified using a QIAquick PCR Purification column and eluted in 30 μ L of water. Two microliters of the reaction were transformed into *E. coli* Electromax™ DH10B™ (Life Technologies, Inc.) cells and plated on LB media containing 25 μ g/mL of
20 kanamycin. Control ligations indicated a background level (vectors with no insert) of less than 3%. Multiple transformations were done to obtain approximately 40,000 colonies. Colonies were scrapped from plates and plasmid DNA prepared using the Qiagen MiniSpin Plasmid procedure. Plasmid DNA was precipitated with ammonium acetate and ethanol to increase its concentration
25 before transformation into selection hosts. Plasmid DNA was also isolated from single colonies and sequenced to obtain an estimate of the mutation rate. The average mutation rate with this method was 1.3 altered nucleotides per Kb.

In a third method, mutagenic PCR was conducted based on the protocol of Cadwell and Joyce (*PCR Methods Appl.* 2:28-33, 1992). This method used various dilutions of a mutagenic buffer containing 21.2 mM MgCl₂, 2.0 mM MnCl₂, 3.2 mM dTTP, and 3.2 mM dCTP. The following
30 volumes of mutagenic buffer were added to separate PCR reactions (each of final volume 100 μ L): 0, 1.56, 3.13, 6.25, 12.5, and 25 μ L, in addition to 1X Taq PCR buffer with 1.5 mM MgCl₂, 0.25 μ M of each primer, 200 μ M of each dNTP, 50 ng of pET-KAM1 template DNA, and 10 units of Taq DNA polymerase (Roche). The PCR program included an initial denaturation at 94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, 54°C for 1 minute, and 72°C for 2.25 minutes; and a final extension at 72°C for 7 minutes.

Following PCR, the reactions were treated to eliminate the Taq polymerase by adding EDTA to a final concentration of 5 mM, SDS to 0.5%, and proteinase K to 50 μ g/mL (Matsumura and Ellington, Mutagenic PCR of Protein-Coding Genes for In Vitro Evolution. *Methods in*

- 45 -

Molecular Biology. Vol 182: In Vitro Mutagenesis, 2nd ed. Ed. J. Braman Hamana. Press Inc. Totowa, NJ, 2001). The reactions were heated to 65°C for 15 minutes and gel purified as described above. The first four treatments produced sufficient PCR product for cloning. PCR product was digested, ligated into pPRO-Nde, and transformed into *E. coli* Electromax™ DH10B™ cells.

5 Plasmid DNA was isolated from single colonies and sequenced to obtain an estimate of the mutation rate. The average mutation rate for treatments 1-4 varied from 0 to 0.47% (0 to 4.7 altered nucleotides per Kb). Multiple transformations were conducted to obtain approximately 50,000 colonies for each selected treatment. Colonies were scrapped from plates and plasmid DNA prepared using a Qiagen MiniSpin Plasmid procedure. Plasmid DNA was precipitated with ammonium acetate and ethanol to increase its concentration before transformation into selection hosts.

10

EXAMPLE 3

in vivo Mutagenesis of a *B. subtilis* KAM gene

To introduce mutations into the *B. subtilis* KAM gene (SEQ ID NO: 3) *in vivo*, pPRO-15 KAM1 was passaged through the *E. coli* XL1Red (Stratagene) mutator strain. Approximately 50 ng of plasmid pPRO-KAM1 was transformed into competent XL1-Red cells as directed by the manufacturer, and transformants plated on LB medium containing 25 µg/ml kanamycin. Approximately 200 transformants selected at random were scrapped off the transformation plates and inoculated into two portions of 5 ml LB broth containing 25 µg/ml kanamycin. One portion was 20 grown overnight at 30°C, the other at 37°C.

A small aliquot of each portion was inoculated into fresh LB broth containing 25 µg/ml kanamycin, while mutagenized plasmid DNA was extracted from 1.5 ml of each culture using the QiaSpin Mini kit (Qiagen). Overnight growth and plasmid DNA extraction was repeated two more times, generating mutagenized plasmid libraries from two different temperatures and three cycles of 25 increasing exposure to the mutator strain. The plasmid DNAs were concentrated by ethanol precipitation prior to transformation into selection strains.

EXAMPLE 4

Construction of *E. coli* ΔpanD::CAT strain

30 To identify genes encoding polypeptides that can perform the alanine 2,3-aminomutase reaction, an efficient screen or selection for the desired activity is needed. Therefore, a selection method was developed by recognizing that *E. coli* uses beta-alanine for the synthesis of pantothenic acid which in turn is a component of coenzyme A (CoA) and of acyl carrier protein (ACP). CoA and ACP are the predominant acyl group carriers in living organisms, and are essential for growth.

35 In *E. coli*, the primary route to beta-alanine is from aspartate in a reaction catalyzed by aspartate decarboxylase (E.C. 4.1.1.11), which is encoded by the *panD* gene (FIG. 3). A functional deletion mutation of *panD* results in beta-alanine auxotrophy and growth inhibition, which can

alleviated by the exogenous addition of pantothenate or beta-alanine, or by the production of beta-alanine from another source.

Two *E. coli* strains were used in the screen, both of which are deficient in beta-alanine synthesis. The strain DV1 (#6865, *E. coli* Genetic Stock Center, New Haven CT; Vallari and Rock, 5 *J. Bacteriol.* 164:136-42, 1985) is an *E. coli* mutant made by chemical mutagenesis, which has host (chromosomal) mutations of both the *panF* and *panD* genes which renders both genes non-functional. The *panF* gene encodes the uptake of pantothenate from the medium, and thus the combination of *panD* and *panF* provides a more stringent requirement for beta-alanine for growth. Therefore, 10 although the DV1 strain was known, its use for selecting cells having alanine 2,3-aminomutase activity was not previously known.

The other selection strain, BW25113 Δ *panD*::CAT, includes a deletion of the *panD* locus, to prevent revertants of the *panD* mutation which would be able to grow without exogenous beta-alanine. This strain, which has an insertion of a chloramphenicol resistance marker conferred by the 15 CAT gene into the *panD* locus, was constructed using the gene inactivation method of Datsenko and Wanner (*Proc. Natl. Acad. Sci. USA* 97: 6640-5, 2000) using *E. coli* strains BW25113/pKD46 and BW 25141/pKD3 for the *E. Coli* Genetic Stock Center.

The CAT gene of pKD3 was amplified using primers
TATCAATTGTTACAGGCGATACATGGCACGCTCGGCGCGTGTAGGCTGGAGCTGCTTC
(SEQ ID NO: 4) and
20 GATGTCGCGGCTGGTGAGTAACCAGCCGAGGGATAACAAACATATGAATATCCTCCTTA
G (SEQ ID NO: 5), where the underlined sequence corresponds to the regions in the *E. coli* chromosome immediately upstream and downstream of the *panD* locus, respectively, and the non-underlined regions are homologous to regions in pKD3 that permit amplification of a fragment containing the CAT gene. The PCR reaction included 30 μ l 10X concentrated PCR buffer (Roche 25 Molecular Biochemicals), plasmid pKD3, 0.2 mM each dNTP, 0.2 μ M each primer, and 15 units Taq polymerase (Roche Molecular Biochemicals) in a final volume of 300 μ l. The PCR reaction was incubated at 95°C for 30 seconds followed by 30 cycles of 95°C for 30 seconds, 45°C for 30 seconds, 72°C for 1 min, then 72°C for 10 min. The PCR product was precipitated with ethanol, digested with *Dpn*I, purified with the QIAquick PCR Purification Kit (Qiagen), and transformed into
30 BW25113/pKD46 expressing the recombination functions. Transformants were plated on LB plates containing 25 μ g/ml chloramphenicol and 5 μ M beta-alanine.

Chloramphenicol-resistant transformations were single-colony purified on non-selective LB medium supplemented with 5 μ M beta-alanine at 43°C, and single colonies tested for retention of chloramphenicol resistance, loss of ampicillin resistance (indicating curing of pKD46), and 35 requirement for beta-alanine for growth on M9-glucose minimal medium. Confirmation of correct insertion of the CAT gene into the *panD* locus was carried out by colony PCR of the resultant Δ *panD*::CAT strain using primers that flank the insertion locus (TTACCGAGCAGCGTTAGAG, SEQ ID NO: 6; and CACCTGGCGGTGACAACCAT, SEQ ID NO: 7). While the wild-type *panD*

locus is expected to yield a PCR product of 713 basepairs, the *ΔpanD::CAT* construct yielded a 1215-basepair product. A derivative of the *ΔpanD::CAT* strain, in which the inserted *CAT* gene is removed by the activity of the FLP recombinase encoded by plasmid pCP20, was constructed as described previously (Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97: 6640-5, 2000). This strain is referred to as *ΔpanD*.

5 A secondary route to beta-alanine exists in *E. coli* based on the reductive pathway of uracil catabolism (West, *Can. J. Microbiol.* 44: 1106-9, 1998, FIG. 2). In this pathway, uracil is reduced to dihydrouracil by the enzyme dihydropyrimidine dehydrogenase (E.C. 1.3.1.2). Dihhydrouracil is then converted by dihydropyrimidinase (E.C. 3.5.2.2) to N-carbamoyl-beta-alanine, which in turn is 10 hydrolyzed by N-carbamoyl-beta-alanine amidohydrolase (E.C. 3.5.1.6) to beta-alanine, CO₂, and NH₃. To prevent the formation of beta-alanine by this pathway, the gene encoding dihydropyrimidine dehydrogenase, *yeiA* (GenBank Accession No. AAC75208), was insertionally deleted by the method of Datsenko and Wanner as described above. The *CAT* gene of pKD3 was 15 amplified using primers
GCGGCGTGAAGTTCCCAACCCGTTCTGCCTCTCTTCTTCGTGTAGGCTGGAGCTGCTTC
(SEQ ID NO: 8), and
TTACAACGTTACCGGGTGTCTTCTCGCCTTCTAAACCATATGAATATCCTCCTTAG
(SEQ ID NO: 9), where the underlined sequence corresponds to the regions in the *E. coli* chromosome immediately upstream and downstream of the *yeiA* locus, respectively, and the non-underlined sequence are homologous to the regions in pKD3 that permit amplification of a fragment 20 containing the *CAT* gene. Chloramphenicol-resistant insertion mutants were isolated as described above, and the resistance marker transduced into the *ΔpanD* strain to generate the double mutant *ΔpanD/ΔyeiA::CAT*.

25 Electrocompetent cells of *E. coli* BW 25115 *ΔpanD::CAT*, *ΔpanD*, or *ΔpanD/ΔyeiA::CAT*, were generated and used as hosts for the transformation of libraries of mutant lysine 2,3-aminomutase DNAs as described in EXAMPLE 6.

EXAMPLE 5

Cloning and *in vitro* Mutagenesis of a *Porphyromonas gingivalis* KAM gene

30 The lysine 2,3-aminomutase gene from *Porphyromonas gingivalis* was amplified by PCR from genomic DNA and cloned into the *NdeI* and *NotI* sites of vector pET22B (Novagen). Mutagenic PCR was conducted by the method of Cadwell and Joyce (*PCR Methods Appl.* 2:28-33, 1992), using T7 promoter and T7 terminator primers for amplification and 6.25 μL or 9.38 μL of mutagenic buffer per 100 μL reaction. The PCR products were gel purified (Qiagen) and digested 35 sequentially with *NdeI* and *NotI*. The digested PCR products were ligated into pPRONde vector and transformed into *E. coli* Electromax™ DH10B™ as described in EXAMPLE 2. Multiple transformations were conducted to obtain at least 60,000 colonies per mutation treatment. Colonies

were scrapped from plates and plasmid DNA was prepared and precipitated to increase its concentration. The resulting libraries had mutation rates of 0.3% and 0.35%.

EXAMPLE 6

5

Identification of Clones having Alanine 2,3-Aminomutase Activity

The mutagenized lysine 2,3-aminomutase plasmid libraries generated above in EXAMPLE 2 was transformed into electrocompetent *E. coli* strain DV1 cells. Transformants were plated on LB containing 25 μ g/ml kanamycin at the appropriate dilution to obtain an estimate of total transformants and on M9 minimal medium supplemented with 0.4% glucose, 0.2% Vitamin Assay Casamino Acids 10 (DIFCO/Becton Dickinson, Sparks, MD), and 25 μ g/ml kanamycin (Sigma, St. Louis, MO). For some selections, IPTG was added to 0.25 mM.

The *ΔpanD::CAT* strain of *E. coli* described in EXAMPLE 4 was transformed in a similar manner with libraries generated in EXAMPLES 2, 3, and 5, except that transformants were plated on M9 minimal medium supplemented with 0.4% glucose, and 25 μ g/ml kanamycin (Sigma, St. Louis, 15 MO). For some selections on *B. subtilis* libraries, IPTG was added to 0.25 mM, $Fe_2(NH_4)_2SO_4$ was added to 50 μ M, and chloroamphenicol was added to 25 μ g/mL. For some selections on *P. gingivalis* libraries, IPTG was added to 50 μ M, $Fe_2(NH_4)_2SO_4$ was added to 50 μ M, chloroamphenicol was added to 25 μ g/mL, L-alanine was added to 1 mg/mL and L-lysine was added to 2 mg/mL.

Transformants growing on the minimal medium plates arose at a frequency of approximately 20 1×10^{-4} relative to the number to total transformants as measured by the number of colonies growing on LB plus 25 μ g/ml kanamycin. Plasmid DNA from the colonies growing on minimal medium was prepared using the Qiagen Miniprep kit and retransformed into the *ΔpanD::CAT* strain of *E. coli* to confirm that the ability to grow in the absence of added beta-alanine was conferred by a function carried by the plasmid. Plasmid DNA was prepared from retransformed colonies and the *kam* gene 25 sequenced to determine any changes relative to the wildtype *B. subtilis* or *P. gingivalis* *kam* gene sequences.

A mutated *B. subtilis* *kam* gene sequence, which encodes for an alanine 2,3-aminomutase, is shown in SEQ ID NO: 20, and the corresponding amino acid sequence shown in SEQ ID NO: 21. The plasmid carrying this sequence is designated pLC4-7LC1. There were three amino acid changes 30 observed in the mutated sequence, as compared to the wildtype *B. subtilis* *kam* gene sequence (FIG. 4). There was a L103M substitution, a M136V substitution, and a D339H substitution in the alanine 2,3-aminomutase protein (where the first amino acid is the wild-type sequence, the number is the amino acid position, and the second amino acid is the sequence observed in the alanine 2,3-aminomutase sequence). The FeS cluster-binding motif (amino acids 134-146 of SEQ ID NO: 21) 35 and the putative PLP-binding motif (amino acids of 288-293 SEQ ID NO: 21) are also shown in FIG. 4. This is the first demonstration of alanine 2,3-aminomutase nucleic acid and amino acid sequences.

A mutated *P. gingivalis* *kam* gene sequence, which encodes for an alanine 2,3-aminomutase, is shown in SEQ ID NO. 29, and the corresponding amino acid sequence shown in SEQ ID NO 30. There were five amino acid changes observed in the mutated sequence, as compared to the wildtype *P. gingivalis* sequence (FIG. 5). There was a N19Y substitution, a L53P substitution, a H85Q 5 substitution, a D331G substitution, and a M342T substitution in the alanine 2,3-aminomutase protein. However, it is possible that not all of these mutations are necessary to have alanine 2,3-aminomutase activity. In aligning the *B. subtilis* and *P. gingivalis* mutant proteins, the *P. gingivalis* D331G substitution is located at the corresponding location within the protein as the *B. subtilis* D339H substitution (FIG. 6), indicating that it may be of particular importance. The FeS cluster-binding motif 10 (amino acids 126-138 of SEQ ID NO: 30) and the putative PLP-binding motif (amino acids 280-285 of SEQ ID NO: 30) are also shown in FIG. 5. This is another demonstration of alanine 2,3-aminomutase nucleic acid and amino acid sequences.

The ability of the mutated *kam* genes to convert alpha-alanine to beta-alanine, and thus to 15 allow production of pantothenate, was determined using liquid growth tests that compare the growth of *ΔpanD* transformants in minimal media containing pantothenate with growth in media lacking pantothenate. The starting inoculum included washed cells from a growing culture, or cells scrapped from a plate.

As an example of using washed cells as inoculum, 3-5 mL cultures were started from single 20 colonies and grown overnight at 30°C in LB broth plus 40 µg/ml kanamycin. The OD₆₀₀ of the cultures were read and equivalent numbers of cells from each culture harvested by centrifugation (approximately 600 total OD x µL, e.g. OD 4.0 x 150 µL). The cells were washed twice with 0.85% NaCl, resuspended in 200 µL 0.85% NaCl, and 30 µL was used to inoculate 3 ml of M9-based minimal media (6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl, 2 mM MgSO₄, 4 g/L 25 glucose, 1 mM CaCl₂, 250 µM IPTG, 40 µg/mL kanamycin, 50 µM Fe(NH₄)₂(SO₄)₂) in glass 13 mm diameter tubes to a starting OD₆₀₀ of about 0.05. Control cultures contained either minimal media supplemented with 20 µM pantothenate, or LB broth with 40 µg/mL kanamycin. Cultures were grown at 37°C without shaking for about 18 hours and the OD₆₀₀ measured.

As shown in Table 1, whereas the cells carrying the vector control grew in minimal medium 30 without added pantothenate or beta-alanine to an average OD₆₀₀ of 0.21 using residual reserves of pantothenate or beta-alanine (approximately 30% of the OD₆₀₀ achieved in the presence of pantothenate), the cells carrying the alanine 2,3-aminomutase clone grew to an average OD₆₀₀ of 0.50, approximately 90% of that achieved in the presence of pantothenate. The addition of Fe²⁺ did not increase the growth of the vector control cells, but allowed the alanine 2,3-aminomutase-bearing 35 cells to achieve growth densities equal to that obtained in the presence of pantothenate or in rich LB broth medium. This indicates that the alanine 2,3-aminomutase gene provides a source of beta-alanine that complements the *panD* mutation.

- 50 -

Table 1: Growth test

Plasmid	Replicate	Medium			
		Minimal	Minimal + Fe ²⁺	Minimal + pantothenate	LB
pPRONde	1	0.206	0.210	0.670	0.647
	2	0.217	0.209	0.683	0.640
pLC4-7LC1	1	0.458	0.550	0.522	0.572
	2	0.460	0.541	0.576	0.552

10

As an example of using plated cultures as inoculum, a colony was scrapped off a plate and resuspended in 50 μ L of minimal media lacking pantothenate. The resuspension (20 μ L) was used to inoculate 1 mL of minimal media in a 1.5 mL microtube and 20 μ L was used to inoculate one mL of minimal media supplemented with pantothenate. The latter culture served as a control for the varying amount of inoculum added. The response of the culture to growth without pantothenate was expressed as a ratio of growth on media lacking pantothenate to growth on media containing pantothenate. Cultures were grown at 25-37°C for 1-3 days with no shaking and OD₆₀₀ measured. A more anaerobic growth test was obtained by filling tubes to a greater extent. This was helpful in testing the *P. gingivalis* mutants.

20

Table 2: Test with scrapped colonies/semi-anaerobic.

	OD ₆₀₀ :M9	OD ₆₀₀ :M9+ pantothenate	Ratio OD ₆₀₀ :M9/OD ₆₀₀ :M9+ pantothenate
<i>Pg aam</i>	0.711	0.791	0.90
<i>Pg aam</i>	0.617	0.77	0.80
<i>Pg aam</i>	0.702	0.811	0.87
<i>Pg aam</i>	0.712	0.879	0.81
<i>Pg aam</i>	0.689	0.843	0.82
<i>Pg aam</i>	0.719	0.851	0.84
pPRONde	0.148	0.824	0.18
<i>Bs aam</i>	0.783	0.801	0.98
<i>Bs aam</i>	0.777	0.838	0.93
<i>Pg kam</i>	0.064	0.792	0.08
<i>Pg kam</i>	0.195	0.876	0.22

Pg aam = Cells with plasmid carrying mutated *P. gingivalis* *kam* gene with alanine 2,3-aminomutase activity

Bs aam = Cells with plasmid carrying mutated *B. subtilis* *kam* gene with alanine 2,3-aminomutase activity

25

Pg kam = Cells with plasmid carrying wildtype *P. gingivalis* *kam* gene

EXAMPLE 7

30

Generation of Individual Mutations in *B. subtilis* Lysine 2,3-Aminomutase

The mutations identified in the wildtype *B. subtilis* lysine 2,3-aminomutase gene above in EXAMPLE 5 were individually constructed in the wildtype *B. subtilis* lysine 2,3-aminomutase gene (SEQ ID NO: 3) using the Stratagene QuikChange™ Site-Directed Mutagenesis Kit. The

- 51 -

oligonucleotides used to generate the L103M mutation were:

CACAAAACAAAATACGATATGGAAGACCCGCTCCATGAGGATGAAGATTCA (SEQ ID NO: 10), and TGAATCTTCATCCTCATGGAGCGGGTCTTCCATATCGTATTTGTTTGTG (SEQ ID NO: 11). The oligonucleotides used to generate the M136V mutation were:

5 GAATCAATGTTCCGTATACTGCCGCTAC (SEQ ID NO: 12), and GTAGCGGCAGTACCGAACATTGATTC (SEQ ID NO: 13). The oligonucleotides to generate the D339H mutation were: GTTCCTACCTTGTACACGCACCAGGCG (SEQ ID NO: 14), and CGCCTGGTGCCTGTACAACAAAGGTAGGAAC (SEQ ID NO: 15).

Using the liquid growth test described in EXAMPLE 6, cells with plasmids carrying the 10 L103M mutation alone were capable of growth in minimal medium without added pantothenate or beta-alanine, however not to the same extent as cells with plasmid pLC4-7LC1, whereas those carrying the M136V or D339H mutations alone had the host *ΔpanD* phenotype. Combinations of the L103M mutation with the M136V and D339H mutations in an otherwise wildtype *B. subtilis* kam sequence yielded a gene that conferred the same ability to grow in the absence of beta-alanine or 15 pantothenate as did pLC4-7LC1, confirming that these three mutations, or a subset of them, are sufficient to confer alanine 2,3-aminomutase activity.

One skilled in the art will understand that alternative substitutions in these positions can be generated. Thus, using oligonucleotides similar to SEQ ID NOS: 10 and 11 in which the codon corresponding to L103 was randomized, mutants with substitutions L103K, L103R, L103E, and 20 L103S were obtained that conferred to the *ΔpanD* strain the ability to grow in the absence of beta-alanine or pantothenate. Further, using oligonucleotides similar to SEQ ID NO: 14 and 15 in which the codon corresponding to D339 was randomized, mutants with substitutions D339Q, D339T, D339N, were obtained that conferred to the *ΔpanD* strain the ability to grow in the absence of beta-alanine or pantothenate.

25

EXAMPLE 8

Selection for Alanine 2,3-Aminomutase Activity Without Using Mutagenized Lysine 2,3-Aminomutase

An alternative method to identifying cells having alanine 2,3-aminomutase activity is to 30 plate cells, such as the *DV1* or *ΔpanD::CAT* cells described above, on the media described above, without transfecting them with the mutagenized lysine 2,3-aminomutase library. Such cells are selected as described above, and verified for the presence of alanine 2,3-aminomutase activity as described in EXAMPLES 6 and 9.

Cells can be mutagenized before plating, for example by exposing the cells to UV irradiation 35 or chemicals (such as MES). This permits isolation of mutants having mutations in one or more other genes which result in the cell having alanine 2,3-aminomutase activity.

Alternatively, the cells can be unaltered before plating (e.g. not transformed, not mutagenized). This method permits isolation of naturally occurring strains having alanine 2,3-aminomutase activity.

5

EXAMPLE 9

Demonstration of Alanine 2,3-Aminomutase Activity

Cells obtained using the screening methods described above were verified for their alanine 2,3-aminomutase activity. For cells that were transformed with a mutagenized library (EXAMPLES 2, 3 and 5), plasmids were isolated from selection host using standard molecular biology methods. 10 The resulting plasmids were retransformed into the selection host, the plasmids reisolated, and the resulting clones sequenced as described in EXAMPLE 6. For un-transformed cells (EXAMPLE 8), the gene conferring the alanine 2,3-aminomutase activity can be cloned, for example using shotgun cloning.

Several assays can be used to assay for alanine 2,3-aminomutase activity, such as measuring 15 biosynthesis of [¹³C]coenzyme A from [3-¹³C]alanine via [3-¹³C]beta-alanine, by using an enzyme assay that measures the conversion of alpha-alanine to beta-alanine, or an assay that measures the presence of beta-alanine in cells or extracts of cells carrying alanine 2,3-aminomutase.

Biosynthesis of [¹³C]coenzyme A from [3-¹³C]alpha-alanine via [3-¹³C]beta-alanine

20 Insertional deletion of the *panD* gene, whose gene product (aspartate 1-decarboxylase) catalyzes the production of beta-alanine from aspartate, results in pantothenate deficiency and hence the inability to produce coenzyme A. However, *ΔpanD* cells possessing an alanine 2,3-aminomutase capable of producing beta-alanine from alpha-alanine would be able to bypass this deficiency; in particular, these cells, when grown in the presence of [3-¹³C]alpha-alanine, would incorporate the 25 [¹³C] label into coenzyme A. This test was used to confirm that the *B. subtilis* alanine 2,3-aminomutase sequence isolated in EXAMPLE 6 (SEQ ID NOS: 20 and 21) could catalyze the conversion of alpha-alanine to beta-alanine.

Cells of *E. coli* *ΔpanD/ΔyeiA::CAT* transformed with pPRONde, pPRO-KAM1, or pLC4-7LC1 were grown overnight at 37°C in minimal medium (EXAMPLE 6) except with 25 µg/ml 30 kanamycin and 10 µM Fe(NH₄)₂(SO₄)₂, and with 1 mM alanine (unlabeled), and 10 µM beta-alanine. The cultures were diluted 100-fold in minimal medium with 25 µg/ml kanamycin, 10 µM 35 Fe(NH₄)₂(SO₄)₂, and 11 mM [3-¹³C]alpha-alanine (99%, Cambridge Isotope Laboratories, Andover, MA) but no unlabeled alpha-alanine or beta-alanine. Following growth at 30°C for approximately 20 hours, the cells were recovered by centrifugation and extracts generated by the method of Jaskowski and Rock (*J. Bacteriol.* 148: 926-32, 1981) in the presence of 10 mM dithiothreitol to convert thioesters of coenzyme A to the free sulphydryl form.

The extracts were analyzed using a Micromass Ultima LC/MS system which included of a Waters 2690 liquid chromatograph with a Waters 996 Photo-Diode Array (PDA) absorbance monitor placed in series between the chromatograph and the triple quadrupole mass spectrometer. LC separations were made using a 4.6 x 150 mm YMC ODS-AQ (3 μ m particles, 120 \AA pores) reversed-phase chromatography column at room temperature. Gradient elution of the analytes was performed using aqueous 25 mM ammonium acetate containing 0.5% (v/v) acetic acid (Buffer A), and acetonitrile containing 0.5% (v/v) acetic acid (Buffer B). The elution was isocratic at 10% B, 0-10 min, then linear from 10% B to 100% B, 10-12 min. The flow rate was 0.250 mL/min and photodiode array UV absorbance was monitored from 200 nm to 400 nm. All parameters of the 5 electrospray MS system were optimized and selected based on generation of protonated molecular ions ($[\text{M} + \text{H}]^+$) of the analytes of interest, and production of characteristic fragment ions. The following instrumental parameters were used for ESI-MS detection of Coenzyme A in the positive 10 ion mode: capillary: 4.0 V; cone: 80 V; hex 1: 25 V; aperture: 0 V; hex 2: 0 V; source temperature: 100°C; desolvation temperature: 350°C; desolvation gas: 500 L/h; cone gas: 40 L/h; low mass 15 resolution: 15.0; high mass resolution: 15.0; ion energy: 0; multiplier: 650. Uncertainties for reported mass/charge ratios (m/z) and molecular masses are $\pm 0.01\%$. The ratio of peak areas with m/z 769 ($[^{13}\text{C}]$ coenzyme A) to peak area with m/z 768 (unlabeled coenzyme A) are shown in Table 3.

Table 3: Biosynthesis of $[^{13}\text{C}]$ Coenzyme A

20

Plasmid or sample	Ratio $m/z=769:m/z=768$
Coenzyme A standard	0.26
pPRONde	0.36
pPRO-KAM1	0.53
25 pLC4-7LC1	1.90

25

The results shown in Table 3 confirm that cells bearing the plasmid carrying the mutant with alanine 2,3-aminomutase activity (SEQ ID NOS: 20 and 21), when grown on $[^{13}\text{C}]$ alpha-alanine, produce a higher ratio of $[^{13}\text{C}]$ coenzyme A to $[^{12}\text{C}]$ coenzyme A compared to normal abundance 30 $[^{13}\text{C}]$ coenzyme A or with cells bearing either the vector or the wildtype *B. subtilis* lysine 2,3-aminomutase gene. This demonstrates that the alanine 2,3-aminomutase sequence can produce beta-alanine, an obligatory intermediate in the biosynthesis of coenzyme A.

Enzyme Assays

35

An enzyme assay which measures the conversion of alpha-alanine to beta-alanine, or which measures for the presence of beta-alanine, can be performed to determine if a cell has alanine 2,3-aminomutase activity. For example, the method described by Chen *et al.* (*Biochem. J.* 348:539-49, 2000) to determine the lysine 2,3-aminomutase activity can be applied to the determination of alanine 2,3-aminomutase activity by substituting L-[U- ^{14}C]alanine for L-[U- ^{14}C]lysine in the incubation with

reductively preincubated enzyme or cell extract, and separation of the radioactive alpha-alanine and beta-alanine by paper electrophoresis followed by scintillation counting of the spots corresponding to alpha-alanine and beta-alanine, respectively. Alternatively, the purified and reductively preincubated alanine 2,3-aminomutase can be incubated with alpha-alanine and the reaction mixture separated by 5 high performance liquid chromatography to separate the product beta-alanine from alpha-alanine and quantify the product (Abe *et al.*, *J. Chromatography B*, 712:43-9, 1998).

The formation of beta-alanine from alpha-alanine can also be monitored in whole cells of the *E. coli* *ΔpanD::CAT* strain transformed with a plasmid expressing an alanine 2,3-aminomutase by incubation of the cells in M9 minimal medium (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989) containing 0.4% (w/v) glucose, 25 µg/ml kanamycin (for a plasmid conferring resistance to kanamycin), 0.25 mM IPTG, and 1mg/ml [¹³C]-labeled alpha-alanine, extracting the cells, and detecting the [¹³C]-beta alanine by high performance liquid chromatography/mass spectrometry using methods known by those skilled in the art.

15

EXAMPLE 10**Synthetic Operons for 3-HP Production from Beta-Alanine**

Biosynthetic pathways that allow production of 3-HP via beta-alanine were generated (FIG. 1). One pathway to 3-HP from beta-alanine involves the use of a polypeptide having CoA transferase activity, that is, an enzyme from a class of enzymes that transfers a CoA group from one metabolite 20 to the other. As shown in FIG. 1, beta-alanine can be converted to beta-alanyl-CoA using a polypeptide having CoA transferase activity and CoA donors such as acetyl-CoA or propionyl-CoA. Alternatively, beta-alanyl-CoA can be generated by the action of a polypeptide having CoA synthetase activity. The beta-alanyl-CoA can be deaminated to form acrylyl-CoA by a polypeptide 25 having beta-alanyl-CoA ammonia lyase activity. The hydration of acrylyl-CoA at the beta position to yield 3-HP-CoA can be carried out by a polypeptide having 3-HP-CoA dehydratase activity. The 3-HP-CoA can act as a CoA donor for beta-alanine, a reaction that can be catalyzed a polypeptide having CoA transferase activity, thus yielding 3-HP as a product. Alternatively, 3-HP-CoA can be 30 hydrolyzed to yield 3-HP by a polypeptide having specific CoA hydrolase activity.

These pathways use several enzymes that were cloned and expressed as described in WO 30 02/42418 (herein incorporated by reference) or below. *Megasphaera elsdenii* cells (ATCC 17753), *Chloroflexus aurantiacus* cells (ATCC 29365), *Clostridium propionicum* (ATCC 25522), *Clostridium acetobutylicum* (ATCC 824), *Pseudomonas aeruginosa* (ATCC 17933), *Bacillus subtilis* (ATCC 23857), *Alcaligenes faecalis* (ATCC 25094), and rat cDNA (Clontech, Palo Alto, CA) were used as sources of DNA. One skilled in the art will understand that similar methods can be used to 35 obtain the sequence of these enzymes from any organism.

Individual genes were cloned, expressed and assayed prior to operon constructions. The synthetic operons for production of 3-HP in *E. coli* were cloned into pET-11a (5.7 kb) expression vector under control of the T7 promoter (Novagen), pPROLar.A (2.6 kb) vector with lac/ara-1

promoter (Clontech, Palo Alto, CA), and pTrc99A (4.2 kb) vector with trc promoter (Pharmacia Biotech, Uppsala, Sweden). Several operons with different combinations of relevant genes were generated as described below. Assays for propionyl-CoA transferase and acrylyl-CoA hydratase (or 3-HP dehydratase) are described in WO 02/42418 (herein incorporated by reference).

5

*Isolation of a 3-HP dehydrogenase gene from *Alcaligenes faecalis* M3A*

A. faecalis (ATCC #700596) is salt marsh bacterium that metabolizes acrylate via 3-HP. The 3-HP produced from acrylate is likely converted to malonic semialdehyde by a 3-HP dehydrogenase. To isolate the gene encoding this dehydrogenase, *A. faecalis* genomic DNA was isolated as follows. A five-mL *A. faecalis* culture was grown at 37°C in trypticase soy broth, and cells harvested then resuspend in 400 mL TE buffer. Subsequently, 20mL of 10% SDS, 100 µl 10 mg/ml proteinase K, and 10 mL 100 mg/ml lysozyme were added to the cell suspension and the mixture incubated for two hours at 42°C with occasional mixing. To this mix, 150 mL phenol was added and the mixture shaken for at least two hours at 37°C, then approximately 800 mL chloroform added. The mixture was mixed by vortexing and centrifuged for 30 minutes at 15000 rpm. The upper aqueous phase was transferred to a clean microfuge tube, and the DNA precipitated with 60 mL 3M NaOAc and approximately 1 mL ethanol, and recovered by spooling. The DNA was resuspended with 400 mL TE buffer and 20 mL 200 mg/ml RNase added, and the mixture incubated for 1 hour at 37°C. The DNA was re-precipitated with NaOAC and ethanol, rinsed several times with 70% ethanol and resuspend in TE buffer.

The following degenerate primers were designed based on conserved regions of publicly known amino acid sequences of the 3-hydroxyisobutrate dehydrogenase genes which are expected to be homologous to the desired 3-HP dehydrogenase. AFHPDHF1: 5'

TTYATYGGBYTSGGBAAYATGGG 3' (SEQ ID NO: 16); AFHPDHF2: 5'

25 GAYGCNCCNGTBWSSGGBGG 3' (SEQ ID NO: 17); and AFHPDHR2: 5'
CATRTTTRTTRCARATYTTNGC 3' (SEQ ID NO: 18). PCR reactions using *A. faecalis* genomic DNA as template were carried out using Taq DNA polymerase (Roche) according to the manufacturer's instructions, using either SEQ ID NOS: 16 and 18 (reaction A) or SEQ ID NOS: 17 and 18 (reaction B). The PCR program consisted of an initial incubation of 94°C for 2 minutes, 4 cycles of 94°C, 30 seconds; 56°C, 45 seconds, 72°C 3 minutes; 4 cycles of 94°C, 30 seconds; 54°C, 45 seconds, 72°C 3 minutes; 4 cycles of 94°C, 30 seconds; 52°C, 45 seconds, 72°C 3 minutes; 4 cycles of 94°C, 30 seconds; 50°C, 45 seconds, 72°C 3 minutes; and 16 cycles of 94°C, 30 seconds; 47°C, 45 seconds, 72°C 3 minutes, followed by a final incubation of 7 minutes at 72°C. Both reactions gave products of approximately 500 bp. The PCR product from reaction A was gel isolated,

30 cloned into pCR11 and transformed into TOP10 chemical competent cells, selected on LB medium containing 50 mg/ml kanamycin. Clones having the right size insert were selected and their plasmids isolated and sequenced. Based on these sequences the following gene specific nested primers were designed: GWHPDF1: 5' GGTITACGAGGGCGAGAACGGCTTGCT 3' (SEQ ID NO: 19);

GWHPDF2: 5' CAAGCTGGTCTGTTCATGCTGGATG 3' (SEQ ID NO: 26); GWHPDR1: 5' AAGCGGTTCTGCCCTCGTAAACCTGA 3' (SEQ ID NO: 27); and GWHPDR2: 5' CGCATTCAAGTCAAAGACGTTCAGGCTA 3' (SEQ ID NO: 32) and the Genome Walk technique used to isolate the entire ORF of the gene encoding the 3-HP dehydrogenase. The sequence of this 5 ORF is shown in SEQ ID NO: 33, and the corresponding protein sequence in SEQ ID NO: 34. The start codon of the 3-HP dehydrogenase is at position 408 of SEQ ID NO: 33 and is preceded by a ribosome-binding site at positions 397-403 of SEQ ID NO: 33. The stop codon is at position 1304 of SEQ ID NO: 33.

10 **Cloning, Expression, and Assay of β -alanine-CoA ammonia lyase (ACL)**

Two *acl* genes were cloned from *C. propionicum*. *acl-1* (SEQ ID NO: 22) encodes a 145 amino acid protein (SEQ ID NO: 23) and *acl-2* (SEQ ID NO: 53) encodes a 144 amino acid protein (SEQ ID NO: 54). These two proteins are highly homologous and differ by only 8 amino acids at the C-terminus. The *acl-1* and *acl-2* genes were cloned using the following primers: OSaclNdeF: 5'- 15 GGGATTCCATATGGTAGGTAAAAAGGTTGTACATC-3' (SEQ ID NO: 35), and OSaclBamR: 5'-CGACGGATCCATTCTGCCGCTTGAATAACT'AAAG-3' (SEQ ID NO. 36) for *acl-1*, and SEQ ID NO: 35 and OSacl2BamR: 5'-CGACGGATCCCAGAAATGTCACCAAAAATTATTGAG-3' (SEQ ID NO: 37) for *acl-2*. The resulting sequences were cloned into the pET11a vector digested with *NdeI* and *BamHI*. Resulting plasmids pACL-1 and pACL-2 were transformed into BL21(DE3) 20 cells. BL21(DE3) carrying pET11a (control), pACL-1 and pACL-2 were grown in 10 ml LB medium supplemented with 50 μ g/ml carbenicillin to OD₆₀₀~0.5 and induced with 100 μ M IPTG for 4 hours. The induced cells were collected by centrifugation at 3500 rpm in Avanti J20 centrifuge (Beckman, Fullerton, CA) and treated with Bug Buster (Novagen, Madison, WI) according to the manufacturer instruction. The resulting cell extract was used in an enzyme assay that followed the 25 conversion of acrylyl-CoA to beta-alanine-CoA (the reverse reaction with respect to the pathway in FIG. 1).

The assay mixture contained 10 μ l 1M TAE, 20 μ l 1M NH₄Cl, 2 μ l 100 μ M acrylyl-CoA, 10 μ l cell extract, and 158 μ l H₂O. The enzymatic reaction was incubated for five minutes at 37°C and stopped by addition of 200 μ l 10% TFA. The mixture was loaded on C18 Sep-Pak Vac 1cc column 30 (Waters, Milford, MA), eluted with 200 μ l 40% acetonitrile, 0.1% TFA and reduced in volume to 100 μ l by centrifugation in SpeedVac (Savant Instruments, Holbrook, NY). Formation of beta-alanyl-CoA was detected by LC-MS using standard methods. Both ACL-1 and ACL-2 enzymes were active and used for beta-alanine operon construction.

35 **Cloning, Expression, and Assay of CoA Transferase from *E. coli***

The open reading frame *yfdE* (identified as a hypothetical protein in the PubMed database) was amplified using PCR. Because the open reading frame had two potential start sites, the following primers were used to clone and express both genes: *yfdE* gtg nde sen (5'-

AGAGAGCATATGTCTTTCACCTTCGGC-3'; SEQ ID NO: 38), and *yfdE* atg *nde* sen (5'-AGAGAGGGATCCCGGGCTCCCACAATGTTGAAATG-3' SEQ ID NO: 39) for *yfdE-1*, and *yfdE* *gtg* *nde* sen (SEQ ID NO: 38) and *yfdE* *bam* anti (5'-AGAGAGCATATGACAAATAATGAAAGCAAAGG-3', SEQ ID NO: 40) for *yfdE-2*.

5 Chromosomal DNA from *E. coli* MG1655 was used as template for PCR performed with *Pfu* Turbo (Stratagene) using the following PCR conditions: 94°C for 5 minutes; 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes 20 seconds, followed by incubation at 72°C for 7 minutes. The PCR reaction was purified using QIAquick PCR purification Kit (Qiagen), digested with *Nde*I and *Bam*HI, cloned into pET28b (Novagen) digested with the same restriction 10 enzymes, and transformed into chemically competent TOP10 cells (Invitrogen). Plasmids from positive clones were isolated and transformed into BL21(DE3) expression cells (Novagen). Cells were grown in LB media at 37°C to an OD₆₀₀ of 0.6 and were induced with 100 μM IPTG and incubated an additional 3 hours after induction. The cells were harvested by centrifugation and washed once with 0.85% NaCl. The cell pellet was stored at -80° C until further use.

15 The cell pellet was thawed on ice, and resuspended in 4 ml binding buffer (Novagen HisBind purification kit). The cells were lysed by three passages through a French Pressure Cell (SLM Aminco) (10000 psi). The cell debris was removed by centrifugation (30,000xg for 30 minutes). The extract was filtered through a 0.45 μm syringe filter before loading on a Quick 900 cartridge following the manufacturer's instructions (Novagen). Purified protein was desalted using a 20 PD-10 column (Pharmacia) according to the manufacturer's instructions. The buffer used was 5 mM boric acid, 5 mM Tris, 5 mM citric acid, 5 mM NaH₂PO₄ pH 7.0.

Purified protein was assayed using a reaction mix containing 100 mM K phosphate, pH 7.0, 100 mM beta-alanine, 1 mM acetyl-CoA, and 20 μl purified CoA transferase in a total assay volume of 200 μl. The reaction was incubated for 20 minutes at room temperature and then stopped with 100 25 μl 10% trifluoroacetic acid (TFA). The reactions were purified using 1 cc SepPak Vac cartridges (Waters Milford, MA) conditioned with 1 ml methanol and washed twice with 1 ml 0.1% TFA. The sample was applied and the cartridge washed twice with 1 ml 0.1% TFA. The sample was eluted with 200 μl 40% acetonitrile containing 0.1% TFA, dried to ½ volume in a rotary evaporator and analyzed by liquid chromatography/mass spectrometry. A peak corresponding to the expected mass 30 for beta-alanyl-CoA was present in assays with *yfdE-1* or *yfdE-2* proteins, and this peak was not present in the controls omitting the purified proteins, indicating that the CoA transferases are responsible for the synthesis of beta-alanyl-CoA.

Operons 1 and 2: ACL - propionyl-CoA transferase - acrylyl-CoA hydratase

35 Operons for the following conversion: beta-alanine to beta-alanyl-CoA to acrylyl-CoA to 3-HP were constructed. A gene encoding CoA transferase was amplified from genomic DNA of *M. elsdenii* by PCR with OSNBpctF (5'-

GGGAATTCCATATGAGAAAAGTAGAAATCATTACAGCTG-3'; SEQ ID NO: 41) and OSHTR

(OSHTR: 5'-ACGTTGATCTCCTCTACATTATTTTCAGTCCCATG-3'; SEQ ID NO: 42) primers.

A CoA hydratase gene was amplified from genomic DNA of *C. aurantiacus* by PCR with OSTHF (5'-CATGGGACTGAAAAATAATGTAGAAGGAGATCAACGT-3'; SEQ ID NO: 43) 5 and OSHBR (5'-CGACGGATCCTCAACGACCACTGAAGTTGG-3'; SEQ ID NO: 44) primers.

ACL-1 and ACL-2 (beta-alanine-CoA ammonia lyase) genes were amplified from *C. propionicum* genomic DNA with primer pairs OsaclXbaF (5'-
CTAGTCTAGAGCTTCTAAGAAACGATTCCG-3'; SEQ ID NO: 45) and OSaclNdeR (5'-
GGGAATTCCATATGCGTAACCTCCTCCTGCTATCATTACCGGGGTGCTTCT-3'; SEQ ID
10 NO: 46) for *acl-1*; and OSacl2XbaF (5'-CTAGTCTAGAGGAAACCGCTAACGAACTC-3'; SEQ
ID NO: 47) and OSacl2-2NdeR (5'-
GGGAATTCCATATGCGTAACCTCCTCCTGCTATTATTGAGGGTGCTTGCATCC-3'; SEQ
ID NO: 48) for *acl-2*.

PCR was conducted in a Perkin Elmer 2400 Thermocycler using Pfu Turbo polymerase 15 (Stratagene) according to the manufacturer instructions. PCR was performed under the following conditions: initial denaturation step 94°C for 2 minutes; 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes; final extention at 72°C for 7 minutes. Resulting PCR products were gel purified using Qiagen Gel Extraction Kit (Qiagen, Inc.).

CoA-transferase and CoA-hydratase PCR products were assembled together in assembly 20 PCR. OSTHF and OSHTR primers (SEQ ID NOS: 42 and 43) are complementary to each other which allowed the complementary DNA ends to anneal to each other during PCR and to extend the DNA in both directions. To ensure the efficiency of the assembly and the following amplification, two end primers OSNBpctF and OSHBR (SEQ ID NOS: 41 and 44) were added to the assembly PCR mixture containing 100 ng of the purified CoA-transferase and CoA-hydratase PCR products and the 25 mix of rTth polymerase (Applied Biosystems, Foster City, CA) and Pfu Turbo polymerase (Stratagene) in a ratio of 8:1. The polymerase mix ensured higher fidelity of the PCR reaction.

Assembly PCR was run under the following conditions: initial denaturation step 94°C for 1 minute; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, 68°C for 2.5 minutes; final extention at 68°C for 7 minutes. The assembled PCR product was gel purified as described above and digested with 30 *NdeI* and *BamHI*. The sites for these restriction enzymes were introduced to assembled PCR product with OSNBpctF (*NdeI*) and OSHBR (*BamHI*) primers (SEQ ID NOS: 41 and 44). The digested PCR product was incubated at 80°C for 30 minutes (to inactivate the restriction enzymes) and used directly for ligation to pET11a vector.

Vector pET11a was digested with *NdeI* and *BamHI*, gel purified using Qiagen Gel 35 Extraction kit, treated with shrimp alkaline phosphatase as suggested by the manufacturer (Roche Molecular Biochemicals) and used for ligation with the assembled PCR product. Ligation was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals). The ligation mixture was transformed into chemically competent NovaBlue cells (Novagen) and plated on LB plates

supplemented with 50 µg/ml carbenicillin. Individual colonies were selected for plasmid DNA purification; plasmid DNA was obtained using Qiagen Spin Miniprep Kit. Plasmids were digested with *NdeI* and *BamHI* and analyzed by gel electrophoresis.

The resulting plasmid was named pTH, digested with *XbaI* and *NdeI*, purified using gel 5 electrophoresis and Qiagen Gel Extraction kit as described above, and used as a vector for consequent cloning of ACL-1 and ACL-2 PCR products digested with the same enzymes. The ligation was performed as described above, and the ligation mixture transformed into chemically competent NovaBlue cells as described above. Individual colonies were selected for plasmid DNA purification; 10 plasmid DNA was obtained using Qiagen Spin Miniprep Kit. Plasmids were digested with *XbaI* and *NdeI* and analyzed by gel electrophoresis. Resulting pATH plasmids carrying the constructed operon were transformed into *E. coli* BL21(DE3) cells to determine the expression of the cloned genes.

To measure the gene expression and 3-HP production, BL21(DE3) cells carrying pATH-1 and pATH-2 plasmids were grown to OD₆₀₀~0.5 in M9CA medium (Difco Laboratories, Sparks, MA) supplemented with 10g/l glucose, 5 g/l beta-alanine and 50 µg/ml carbenicillin, and induced 15 with 100 µM IPTG under aerobic conditions. BL21(DE3) cells carrying pET11a vector served as a control. Cell samples were taken 2 and 4 hours after IPTG induction for polyacrylamide gel electrophoresis analysis. All three enzymes were expressed as shown by the appropriate sized band on the gel. Production of 3-HP from beta-alanine was detected with both operon constructs, pATH-1 and pATH-2, but not in the control cells by LC-MS analysis.

20

Operon 3: 4-aminobutyrate aminotransferase - 3-hydroxyisobutyrate dehydrogenase

In an alternative or additional pathway, beta-alanine can be deaminated by a polypeptide having beta-alanine-2-oxoglutarate aminotransferase activity to yield malonate semialdehyde, which can be further reduced to 3-HP by a polypeptide having 3-HP dehydrogenase activity or a 25 polypeptide having 3-hydroxyisobutyrate dehydrogenase activity.

Methods for isolating, sequencing, expressing, and testing the activity of such polypeptides are described in WO 02/42418 (herein incorporated by reference). One skilled in the art will understand that similar methods can be used to obtain the sequence of any such polypeptide from any organism.

30

The gene encoding 4-aminobutyrate aminotransferase was amplified from genomic DNA of *C. acetobutylicum* by PCR with OsabatF (5'-CCGGAATTCTTTAATATGCGATTGGAGGAG-3'; SEQ ID NO: 49) and OSDATR (5'-GTCCGTCTCCCTTCAGCTTAAATCGCTATTCTTATAGC-3'; SEQ ID NO: 50) primers. A gene encoding 3-hydroxyisobutyrate dehydrogenase was amplified from genomic DNA of *P. aeruginosa* by PCR with OSATDF (5'-

35

GCTATAAGAATAGCGATTTAAGCTGAAAGGGAGACGGAC-3'; SEQ ID NO: 51) and OSibdR (5'-CGACGGATCCGAGTGAGTGAGCCTGGAG-3'; SEQ ID NO: 52) primers. PCR was conducted in a Perkin Elmer 2400 Thermocycler using Pfu Turbo polymerase according to the manufacturer instructions under the following conditions: initial denaturation step 94°C for 2

- 60 -

minutes; 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 1.5 minutes; final extention at 72°C for 10 minutes. Resulting PCR products were gel purified using Qiagen Gel Extraction Kit.

PCR products of 4-aminobutyrate aminotransferase and 3-hydroxyisobutyrate dehydrogenase were assembled together in assembly PCR. The primers shown in SEQ ID NOS: 50 and 51 are complementary to each other and therefore complementary DNA ends could anneal to each other during PCR reaction and to extend the DNA in both directions. To ensure the efficiency of the assembly and the following amplification, two end primers OSabatF and OSibdR (SEQ ID NOS: 49 and 52) were added to the assembly PCR mixture containing 100 ng of the purified 4-aminobutyrate aminotransferase and 3-hydroxyisobutyrate dehydrogenase PCR products and the mix of rTth polymerase and Pfu Turbo polymerase in 8:1 ratio. Assembly PCR was run under the following conditions: initial denaturation step 94°C for 1 minute; 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 3 minutes; final extention at 68°C for 7 minutes. The assembled PCR product was gel purified as described above and digested with *EcoRI* and *BamHI*. The sites for these restriction enzymes were introduced to assembled PCR product with OSabatF (*EcoRI*) (SEQ ID NO: 49) and OSibdR (*BamHI*) (SEQ ID NO: 52) primers. The digested PCR product was heated at 80°C for 30 minutes, gel purified using Qiagen Gel Extraction kit, and used for ligation to pPROLar.A vector.

pPROLar.A was digested with *EcoRI* and *BamHI*, gel purified using Qiagen Gel Extraction kit, treated with shrimp alkaline phosphatase as suggested by the manufacturer and used for ligation with the assembled PCR product. The ligation was performed as described above and transformed into chemically competent TOP10 cells (Novagen) and plated on LB plates supplemented with 25 µg/ml kanamycin. Individual colonies were selected for plasmid DNA purification; plasmid DNA was obtained using Qiagen Spin Miniprep Kit. Plasmids were digested with *EcoRI* and *BamHI* and analyzed by gel electrophoresis. The resultant plasmid carrying the 4-aminobutyrate aminotransferase and 3-hydroxyisobutyrate dehydrogenase genes was designated pATD.

To observe gene expression and 3-HP production, TOP 10 cells carrying pATD plasmids or without plasmids (control) were grown to OD₆₀₀~0.5 in LB medium supplemented with 5 g/l glucose, 5 g/l beta-alanine and 25 µg/ml kanamycin and induced with 100 µM IPTG and 0.5% arabinose under aerobic conditions. Production of 3-HP from beta-alanine was detected with cells carrying pATD, but not in the control cells by LC-MS analysis. 3-HP was observed in cell supernatants after 6 and 24 hours of IPTG induction.

EXAMPLE 11

Synthetic Operons for 3-HP Production from Alpha-Alanine

Several operons were generated in EXAMPLE 10 which permit production of 3-HP via beta-alanine through several alternative pathways. The methods disclosed in this example expand on that, by including the disclosed alanine 2,3-aminomutase sequences disclosed herein in an operon. This allows production of 3-HP via alpha-alanine.

Operon 4: alpha-alanine aminomutase – ACL - propionyl-CoA transferase - acrylyl-CoA hydratase

An operon for the conversion of alpha-alanine to beta-alanine to beta-alanyl-CoA to acrylyl-CoA to 3-HP was constructed as follows. Plasmid pLC4-7LC1 plasmid carrying alanine 2,3-

5 aminomutase (EXAMPLE 6; SEQ ID NOS: 20 and 21) was used for the construction of pLCATH2-1. The ATH-2 operon was amplified from pATH-2 (EXAMPLE 10) with the following primers:

OSacl2NotF2: 5'-AAGGAAAAAAGCGGCCGCAGATTAAAGGAGGAATTCTCAATGG-3' (SEQ ID NO: 55) and OShydXbaR: 5'-CTAGTCTAGATCAACGACCCTGAAGTTGG-3' (SEQ ID NO: 56). PCR was conducted as described above using the mix of rTth polymerase and Pfu Turbo

10 polymerase in 8:1 ratio under the following conditions: initial denaturation step 94°C for 2 minutes; 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 68°C for 2 minutes; final extention at 68°C for 7 minutes.

The resulting PCR product was purified using Qiagen PCR Purification Kit and digested with *NotI* and *XbaI*. Digested DNA was heated at 65°C for 30 minutes for enzyme inactivation, gel

15 purified using Qiagen Gel Extraction Kit, and cloned into pLC4-7LC-1 plasmid digested with the same enzymes. The ligation was performed at 16°C overnight using T4 ligase. The ligation mixture was transformed into chemically competent Tuner cells (Novagen) and plated on LB plates

supplemented with 25 µg/ml kanamycin. Individual colonies were selected for plasmid DNA

purification; plasmid DNA was obtained using Qiagen Spin Miniprep Kit. Plasmids were digested

20 with *NotI* and *XbaI* and analyzed by gel electrophoresis. Resulting Tuner (pLCATH2-1) cells were used to observe expression of the cloned genes and production of 3-HP from alpha-alanine and beta-alanine.

Operon 5: α-alanine aminomutase - 4-aminobutyrate aminotransferase - 3-hydroxyisobutyrate dehydrogenase

An operon for the conversion of alpha-alanine to beta-alanine to malonic semialdehyde to 3-HP was constructed as follows. Plasmid pLC4-7LC1 (EXAMPLE 6) carrying alanine 2,3-

aminomutase (EXAMPLE 6; SEQ ID NOS: 20 and 21) was used for the construction of pLCATD1.

The ATD operon was amplified from pATD plasmid (EXAMPLE 10) with: OSabatNotF: 5'-

30 AAGGAAAAAAGCGGCCGTTAACATATGCGATTGGAGGAG-3' (SEQ ID NO: 57) and OsibdXbaR: 5'-CTAGTCTAGAGCAGTGAGTGAGCCTGGAG-3' (SEQ ID NO: 58). PCR was conducted as described above for operon 4, and the resulting PCR product purified, digested with *NotI* and *XbaI*, cloned into pLC4-7LC-1 plasmid, transformed into chemically competent Tuner cells, and individual colonies selected as described for Operon 4.

35

Induction of operons and 3-HP production

To observe gene expression and 3-HP production, Tuner cells carrying pLCATH2-1, pLCATD1 plasmids or pPROLar vector (control) were grown to OD₆₀₀~0.5 in LB medium

supplemented with 5g/l glucose, 5g/l alpha-alanine or 5 g/l beta-alanine and 25 μ g/ml kanamycin and induced with 100 μ M IPTG and 0.5% arabinose under aerobic conditions. Production of 3-HP from beta-alanine was detected with cells carrying pLCATH2-1 and pLCATD1, but not in the control cells by LC-MS analysis. 3-HP was observed in cell supernatants after 22 hours of induction.

5

Operon 6: Alanine aminomutase - beta-alanine aminotransferase - 3-hp dehydrogenase – alpha-alanine aminotransferase

An operon for the conversion of pyruvate to alpha-alanine to beta-alanine to malonic semialdehyde to 3-HP was constructed as follows. The gene encoding for alanine 2,3-aminomutase was amplified from pLC4-7LC1 by PCR with KAM10F (5'-CACACAGAATTCAATTAAAGAGGAG-3'; SEQ ID NO: 59) and KAMRBATR 5'-CATAATCAAACCTCAAAGTCAACCATAAGATCTCCTCCTTACTTCATGAAGAATCCCT CC-3'; SEQ ID NO: 60) primers. A beta-alanine aminotransferase gene was amplified from rat cDNA by PCR with KAMRBATF (5'-

10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 160 165 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300 305 310 315 320 325 330 335 340 345 350 355 360 365 370 375 380 385 390 395 400 405 410 415 420 425 430 435 440 445 450 455 460 465 470 475 480 485 490 495 500 505 510 515 520 525 530 535 540 545 550 555 560 565 570 575 580 585 590 595 600 605 610 615 620 625 630 635 640 645 650 655 660 665 670 675 680 685 690 695 700 705 710 715 720 725 730 735 740 745 750 755 760 765 770 775 780 785 790 795 800 805 810 815 820 825 830 835 840 845 850 855 860 865 870 875 880 885 890 895 900 905 910 915 920 925 930 935 940 945 950 955 960 965 970 975 980 985 990 995 1000 1005 1010 1015 1020 1025 1030 1035 1040 1045 1050 1055 1060 1065 1070 1075 1080 1085 1090 1095 1100 1105 1110 1115 1120 1125 1130 1135 1140 1145 1150 1155 1160 1165 1170 1175 1180 1185 1190 1195 1200 1205 1210 1215 1220 1225 1230 1235 1240 1245 1250 1255 1260 1265 1270 1275 1280 1285 1290 1295 1300 1305 1310 1315 1320 1325 1330 1335 1340 1345 1350 1355 1360 1365 1370 1375 1380 1385 1390 1395 1400 1405 1410 1415 1420 1425 1430 1435 1440 1445 1450 1455 1460 1465 1470 1475 1480 1485 1490 1495 1500 1505 1510 1515 1520 1525 1530 1535 1540 1545 1550 1555 1560 1565 1570 1575 1580 1585 1590 1595 1600 1605 1610 1615 1620 1625 1630 1635 1640 1645 1650 1655 1660 1665 1670 1675 1680 1685 1690 1695 1700 1705 1710 1715 1720 1725 1730 1735 1740 1745 1750 1755 1760 1765 1770 1775 1780 1785 1790 1795 1800 1805 1810 1815 1820 1825 1830 1835 1840 1845 1850 1855 1860 1865 1870 1875 1880 1885 1890 1895 1900 1905 1910 1915 1920 1925 1930 1935 1940 1945 1950 1955 1960 1965 1970 1975 1980 1985 1990 1995 2000 2005 2010 2015 2020 2025 2030 2035 2040 2045 2050 2055 2060 2065 2070 2075 2080 2085 2090 2095 2100 2105 2110 2115 2120 2125 2130 2135 2140 2145 2150 2155 2160 2165 2170 2175 2180 2185 2190 2195 2200 2205 2210 2215 2220 2225 2230 2235 2240 2245 2250 2255 2260 2265 2270 2275 2280 2285 2290 2295 2300 2305 2310 2315 2320 2325 2330 2335 2340 2345 2350 2355 2360 2365 2370 2375 2380 2385 2390 2395 2400 2405 2410 2415 2420 2425 2430 2435 2440 2445 2450 2455 2460 2465 2470 2475 2480 2485 2490 2495 2500 2505 2510 2515 2520 2525 2530 2535 2540 2545 2550 2555 2560 2565 2570 2575 2580 2585 2590 2595 2600 2605 2610 2615 2620 2625 2630 2635 2640 2645 2650 2655 2660 2665 2670 2675 2680 2685 2690 2695 2700 2705 2710 2715 2720 2725 2730 2735 2740 2745 2750 2755 2760 2765 2770 2775 2780 2785 2790 2795 2800 2805 2810 2815 2820 2825 2830 2835 2840 2845 2850 2855 2860 2865 2870 2875 2880 2885 2890 2895 2900 2905 2910 2915 2920 2925 2930 2935 2940 2945 2950 2955 2960 2965 2970 2975 2980 2985 2990 2995 3000 3005 3010 3015 3020 3025 3030 3035 3040 3045 3050 3055 3060 3065 3070 3075 3080 3085 3090 3095 3100 3105 3110 3115 3120 3125 3130 3135 3140 3145 3150 3155 3160 3165 3170 3175 3180 3185 3190 3195 3200 3205 3210 3215 3220 3225 3230 3235 3240 3245 3250 3255 3260 3265 3270 3275 3280 3285 3290 3295 3300 3305 3310 3315 3320 3325 3330 3335 3340 3345 3350 3355 3360 3365 3370 3375 3380 3385 3390 3395 3400 3405 3410 3415 3420 3425 3430 3435 3440 3445 3450 3455 3460 3465 3470 3475 3480 3485 3490 3495 3500 3505 3510 3515 3520 3525 3530 3535 3540 3545 3550 3555 3560 3565 3570 3575 3580 3585 3590 3595 3600 3605 3610 3615 3620 3625 3630 3635 3640 3645 3650 3655 3660 3665 3670 3675 3680 3685 3690 3695 3700 3705 3710 3715 3720 3725 3730 3735 3740 3745 3750 3755 3760 3765 3770 3775 3780 3785 3790 3795 3800 3805 3810 3815 3820 3825 3830 3835 3840 3845 3850 3855 3860 3865 3870 3875 3880 3885 3890 3895 3900 3905 3910 3915 3920 3925 3930 3935 3940 3945 3950 3955 3960 3965 3970 3975 3980 3985 3990 3995 4000 4005 4010 4015 4020 4025 4030 4035 4040 4045 4050 4055 4060 4065 4070 4075 4080 4085 4090 4095 4100 4105 4110 4115 4120 4125 4130 4135 4140 4145 4150 4155 4160 4165 4170 4175 4180 4185 4190 4195 4200 4205 4210 4215 4220 4225 4230 4235 4240 4245 4250 4255 4260 4265 4270 4275 4280 4285 4290 4295 4300 4305 4310 4315 4320 4325 4330 4335 4340 4345 4350 4355 4360 4365 4370 4375 4380 4385 4390 4395 4400 4405 4410 4415 4420 4425 4430 4435 4440 4445 4450 4455 4460 4465 4470 4475 4480 4485 4490 4495 4500 4505 4510 4515 4520 4525 4530 4535 4540 4545 4550 4555 4560 4565 4570 4575 4580 4585 4590 4595 4600 4605 4610 4615 4620 4625 4630 4635 4640 4645 4650 4655 4660 4665 4670 4675 4680 4685 4690 4695 4700 4705 4710 4715 4720 4725 4730 4735 4740 4745 4750 4755 4760 4765 4770 4775 4780 4785 4790 4795 4800 4805 4810 4815 4820 4825 4830 4835 4840 4845 4850 4855 4860 4865 4870 4875 4880 4885 4890 4895 4900 4905 4910 4915 4920 4925 4930 4935 4940 4945 4950 4955 4960 4965 4970 4975 4980 4985 4990 4995 5000 5005 5010 5015 5020 5025 5030 5035 5040 5045 5050 5055 5060 5065 5070 5075 5080 5085 5090 5095 5100 5105 5110 5115 5120 5125 5130 5135 5140 5145 5150 5155 5160 5165 5170 5175 5180 5185 5190 5195 5200 5205 5210 5215 5220 5225 5230 5235 5240 5245 5250 5255 5260 5265 5270 5275 5280 5285 5290 5295 5300 5305 5310 5315 5320 5325 5330 5335 5340 5345 5350 5355 5360 5365 5370 5375 5380 5385 5390 5395 5400 5405 5410 5415 5420 5425 5430 5435 5440 5445 5450 5455 5460 5465 5470 5475 5480 5485 5490 5495 5500 5505 5510 5515 5520 5525 5530 5535 5540 5545 5550 5555 5560 5565 5570 5575 5580 5585 5590 5595 5600 5605 5610 5615 5620 5625 5630 5635 5640 5645 5650 5655 5660 5665 5670 5675 5680 5685 5690 5695 5700 5705 5710 5715 5720 5725 5730 5735 5740 5745 5750 5755 5760 5765 5770 5775 5780 5785 5790 5795 5800 5805 5810 5815 5820 5825 5830 5835 5840 5845 5850 5855 5860 5865 5870 5875 5880 5885 5890 5895 5900 5905 5910 5915 5920 5925 5930 5935 5940 5945 5950 5955 5960 5965 5970 5975 5980 5985 5990 5995 6000 6005 6010 6015 6020 6025 6030 6035 6040 6045 6050 6055 6060 6065 6070 6075 6080 6085 6090 6095 6100 6105 6110 6115 6120 6125 6130 6135 6140 6145 6150 6155 6160 6165 6170 6175 6180 6185 6190 6195 6200 6205 6210 6215 6220 6225 6230 6235 6240 6245 6250 6255 6260 6265 6270 6275 6280 6285 6290 6295 6300 6305 6310 6315 6320 6325 6330 6335 6340 6345 6350 6355 6360 6365 6370 6375 6380 6385 6390 6395 6400 6405 6410 6415 6420 6425 6430 6435 6440 6445 6450 6455 6460 6465 6470 6475 6480 6485 6490 6495 6500 6505 6510 6515 6520 6525 6530 6535 6540 6545 6550 6555 6560 6565 6570 6575 6580 6585 6590 6595 6600 6605 6610 6615 6620 6625 6630 6635 6640 6645 6650 6655 6660 6665 6670 6675 6680 6685 6690 6695 6700 6705 6710 6715 6720 6725 6730 6735 6740 6745 6750 6755 6760 6765 6770 6775 6780 6785 6790 6795 6800 6805 6810 6815 6820 6825 6830 6835 6840 6845 6850 6855 6860 6865 6870 6875 6880 6885 6890 6895 6900 6905 6910 6915 6920 6925 6930 6935 6940 6945 6950 6955 6960 6965 6970 6975 6980 6985 6990 6995 7000 7005 7010 7015 7020 7025 7030 7035 7040 7045 7050 7055 7060 7065 7070 7075 7080 7085 7090 7095 7100 7105 7110 7115 7120 7125 7130 7135 7140 7145 7150 7155 7160 7165 7170 7175 7180 7185 7190 7195 7200 7205 7210 7215 7220 7225 7230 7235 7240 7245 7250 7255 7260 7265 7270 7275 7280 7285 7290 7295 7300 7305 7310 7315 7320 7325 7330 7335 7340 7345 7350 7355 7360 7365 7370 7375 7380 7385 7390 7395 7400 7405 7410 7415 7420 7425 7430 7435 7440 7445 7450 7455 7460 7465 7470 7475 7480 7485 7490 7495 7500 7505 7510 7515 7520 7525 7530 7535 7540 7545 7550 7555 7560 7565 7570 7575 7580 7585 7590 7595 7600 7605 7610 7615 7620 7625 7630 7635 7640 7645 7650 7655 7660 7665 7670 7675 7680 7685 7690 7695 7700 7705 7710 7715 7720 7725 7730 7735 7740 7745 7750 7755 7760 7765 7770 7775 7780 7785 7790 7795 7800 7805 7810 7815 7820 7825 7830 7835 7840 7845 7850 7855 7860 7865 7870 7875 7880 7885 7890 7895 7900 7905 7910 7915 7920 7925 7930 7935 7940 7945 7950 7955 7960 7965 7970 7975 7980 7985 7990 7995 8000 8005 8010 8015 8020 8025 8030 8035 8040 8045 8050 8055 8060 8065 8070 8075 8080 8085 8090 8095 8100 8105 8110 8115 8120 8125 8130 8135 8140 8145 8150 8155 8160 8165 8170 8175 8180 8185 8190 8195 8200 8205 8210 8215 8220 8225 8230 8235 8240 8245 8250 8255 8260 8265 8270 8275 8280 8285 8290 8295 8300 8305 8310 8315 8320 8325 8330 8335 8340 8345 8350 8355 8360 8365 8370 8375 8380 8385 8390 8395 8400 8405 8410 8415 8420 8425 8430 8435 8440 8445 8450 8455 8460 8465 8470 8475 8480 8485 8490 8495 8500 8505 8510 8515 8520 8525 8530 8535 8540 8545 8550 8555 8560 8565 8570 8575 8580 8585 8590 8595 8600 8605 8610 8615 8620 8625 8630 8635 8640 8645 8650 8655 8660 8665 8670 8675 8680 8685 8690 8695 8700 8705 8710 8715 8720 8725 8730 8735 8740 8745 8750 8755 8760 8765 8770 8775 8780 8785 8790 8795 8800 8805 8810 8815 8820 8825 8830 8835 8840 8845 8850 8855 8860 8865 8870 8875 8880 8885 8890 8895 8900 8905 8910 8915 8920 8925 8930 8935 8940 8945 8950 8955 8960 8965 8970 8975 8980 8985 8990 8995 9000 9005 9010 9015 9020 9025 9030 9035 9040 9045 9050 9055 9060 9065 9070 9075 9080 9085 9090 9095 9100 9105 9110 9115 9120 9125 9130 9135 9140 9145 9150 9155 9160 9165 9170 9175 9180 9185 9190 9195 9200 9205 9210 9215 9220 9225 9230 9235 9240 9245 9250 9255 9260 9265 9270 9275 9280 9285 9290 9295 9300 9305 9310 9315 9320 9325 9330 9335 9340 9345 9350 9355 9360 9365 9370 9375 9380 9385 9390 9395 9400 9405 9410 9415 9420 9425 9430 9435 9440 9445 9450 9455 9460 9465 9470 9475 9480 9485 9490 9495 9500 9505 9510 9515 9520 9525 9530 9535 9540 9545 9550 9555 9560 9565 9570 9575 9580 9585 9590 9595 9600 9605 9610 9615 9620 9625 9630 9635 9640 9645 9650 9655 9660 9665 9670 9675 9680 9685 9690 9695 9700 9705 9710 9715 9720 9725 9730 9735 9740 9745 9750 9755 9760 9765 9770 9775 9780 9785 9790 9795 9800 9805 9810 9815 9820 9825 9830 9835 9840 9845 9850 9855 9860 9865 9870 9875 9880 9885 9890 9895 9900 9905 9910 9915 9920 9925 9930 9935 9940 9945 9950 9955 9960 9965 9970 9975 9980 9985 9990 9995 9999 10000 10005 10010 10015 10020 10025 10030 10035 10040 10045 10050 10055 10060 10065 10070 10075 10080 10085 10090 10095 10099 10100 10101 10102 10103 10104 10105 10106 10107 10108 10109 10110 10111 10112 10113 10114 10115 10116 10117 10118 10119 10120 10121 10122 10123 10124 10125 10126 10127 10128 10129 10130 10131 10132 10133 10134 10135 10136 10137 10138 10139 10140 10141 10142 10143 10144 10145 10146 10147 10148 10149 10150 10151 10152 10153 10154 10155 10156 10157 10158 10159 10160 10161 10162 10163 10164 10165 10166 10167 10168 10169 10170 10171 10172 10173 10174 10175 10176 10177 10178 10179 10180 10181 10182 10183 10184 10185 10186 10187 10188 10189 10190 10191 10192 10193 10194 10195 10196 10197 10198 10199 10200 10201 10202 10203 10204 10205 10206 10207 10208 10209 10210 10211 10212 10213 10214 10215 10216 10217 10218 10219 10220 10221 10222 10223 10224 10225 10226 10227 10228 10229 10230 10231 10232 10233 10234 10235 10236 10237 10238 10239 10240 10241 10242 10243 1

the assembly PCR mixture containing 100 ng of two purified alanine aminomutase and beta-alanine aminotransferase PCR products and the mix of rTth polymerase and Pfu Turbo polymerase in a ratio of 8:1. Other two end primers, SEQ ID NOS: 63 and 66 were added to the assembly PCR mixture containing 100 ng of purified 3-HP dehydrogenase and alpha-alanine aminotransferase, and the mix 5 of rTth polymerase and Pfu Turbo polymerase in a ratio of 8:1. Assembly PCR was run under the following conditions: initial denaturation step 94°C for 2 minutes; 5 cycles of 94°C for 30 seconds, 48°C for 30 seconds, 68°C for 4 minutes; 5 cycles of 94°C for 30 seconds, 52°C for 30 seconds, 68°C for 4 minutes; 5 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 4 minutes; 10 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 68°C for 4 minutes; final extention at 68°C for 7 minutes.

10 A second assembly PCR was performed to combine the assembled pairs to make Operon 6 which contained all four genes. Two end primers (SEQ ID NOS: 59 and 66) were added to the PCR mixture containing 100 ng of the purified pair of alanine aminomutase/beta-alanine aminotransferase; 100 ng of the purified pair of 3-HP dehydrogenase/alpha-alanine aminotransferase, and the mix of rTth polymerase and Pfu Turbo polymerase in a ratio of 8:1. The assembly PCR was run under the 15 following conditions: initial denaturation step 94°C for 2 minutes; 15 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 70°C for 5 minutes; 10 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 70°C for 5 minutes; final extention at 70°C for 7 minutes.

The assembled PCR product was gel purified as described above and digested with *EcoRI* and *EcoRV*. The sites for these restriction enzymes were introduced to assembled PCR product with 20 SEQ ID NO: 59 (*EcoRI*) and SEQ ID NO: 66 (*EcoRV*) primers. The digested PCR product was heated at 65°C for 30 minutes, gel purified using Qiagen Gel Extraction kit and used for ligation to pTrc99A vector digested with *EcoRI* and *SmaI*. The ligation was performed at 16°C overnight using T4 ligase, and the mixture transformed into chemically competent Tuner cells and plated on LB plates supplemented with 50 µg/ml carbenicillin. Individual colonies were selected for plasmid DNA 25 purification; plasmid DNA was obtained using Qiagen Spin Miniprep Kit. Plasmids were screened by PCR with SEQ ID NOS: 59 and 66 primers and analyzed by gel electrophoresis. The resulting plasmid was named pTrcβ-ala.

Tuner(pTrcβ-ala) cells were used to determine the expression of the cloned genes and production of 3-HP from glucose, alpha-alanine and beta-alanine. Tuner(pTrc99A) were used as a 30 control. Cells were grown to OD₆₀₀~0.5 in M9CA medium (Difco Laboratories) supplemented with 5 g/l glucose and 50 µg/ml carbenicillin; or 5g/l glucose, 5g/l alpha-alanine and 50 µg/ml carbenicillin; or 5 g/l glucose, 5 g/l beta-alanine and 50 µg/ml carbenicillin; and induced with 100 µM IPTG and 0.5% arabinose under aerobic conditions. Production of 3-HP from beta-alanine was detected with cells carrying pTrcβ-ala, but not in the control cells by LC-MS analysis. 3-HP was observed in cell 35 supernatants after 22 hours of induction.

EXAMPLE 12**Production of Pantothenate from Beta-Alanine**

Pantothenate can be produced from beta-alanine by a polypeptides having alpha-ketopantoate hydroxymethyltransferase (E.C. 2.1.2.11), alpha-ketopantoate reductase (E.C. 1.1.1.169), and pantothenate synthase (E.C. 6.3.2.1) activity (FIG. 3).

Using the cloning methods described in EXAMPLES 10 and 11, alpha-ketopantoate hydroxymethyltransferase (E.C. 2.1.2.11), alpha-ketopantoate reductase (E.C. 1.1.1.169), and pantothenate synthase (E.C. 6.3.2.1) polypeptides can be isolated, sequenced, expressed, and tested. One skilled in the art will understand that similar methods can be used to obtain the sequence of any such polypeptides from any organism.

EXAMPLE 13**Recombinant Expression**

With publicly available enzyme cDNA and amino acid sequences, and the enzymes and sequences disclosed herein, such as alanine 2,3-aminomutase, CoA transferase, beta-alanyl-CoA ammonia lyase, 3-HP-CoA dehydratase, 4-aminobutyrate aminotransferase, beta-alanine-2-oxo-glutarate aminotransferase, 3-hydroxypropionate dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, glutamate dehydrogenase, 3-HP-CoA hydrolase, 3-hydroxyisobutryl-CoA hydrolase, poly hydroxyacid synthase, lipase, esterase, CoA hydrolase, alpha-ketopantoate hydroxymethyltransferase, alpha-ketopantoate reductase, pantothenate synthase, pantothenate kinase, 4'-phosphopantethenoyl-1-cysteine synthetase, 4'-phosphopantethenoylcysteine decarboxylase, ATP:4'-phosphopantetheine adenyltransferase, dephospho-CoA kinase acetylating aldehyde:NAD(+) oxidoreductase, alcohol:NAD(+) oxidoreductase, aldehyde dehydrogenase (NAD(P)+) and alcohol dehydrogenase, as well as variants, polymorphisms, mutants, fragments and fusions thereof, the expression and purification of any protein, such as an enzyme, by standard laboratory techniques is enabled. One skilled in the art will understand that enzymes and fragments thereof can be produced recombinantly in any cell or organism of interest, and purified prior to use, for example prior to production of 3-HP, pantothenate and derivatives thereof.

Methods for producing recombinant proteins are well known in the art. Therefore, the scope of this disclosure includes recombinant expression of any protein or fragment thereof, such as an enzyme. For example, see U.S. Patent No: 5,342,764 to Johnson *et al.*; U.S. Patent No: 5,846,819 to Pausch *et al.*; U.S. Patent No: 5,876,969 to Fleer *et al.* and Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, Ch. 17).

Briefly, partial, full-length, or variant cDNA sequences, which encode for a protein or peptide, can be ligated into an expression vector, such as a bacterial expression vector. Proteins and/or peptides can be produced by placing a promoter upstream of the cDNA sequence. Examples of promoters include, but are not limited to *lac*, *trp*, *tac*, *trc*, major operator and promoter regions of phage lambda, the control region of fd coat protein, the early and late promoters of SV40, promoters

- 65 -

derived from polyoma, adenovirus, retrovirus, baculovirus and simian virus, the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, the promoter of the yeast alpha-mating factors and combinations thereof.

5 Vectors suitable for the production of intact native proteins include pKC30 (Shimatake and Rosenberg, 1981, *Nature* 292:128), pKK177-3 (Amann and Brosius, 1985, *Gene* 40:183) and pET-3 (Studier and Moffatt, 1986, *J. Mol. Biol.* 189:113). A DNA sequence can be transferred to other cloning vehicles, such as other plasmids, bacteriophages, cosmids, animal viruses and yeast artificial chromosomes (YACs) (Burke *et al.*, 1987, *Science* 236:806-12). These vectors can be introduced into a variety of hosts including somatic cells, and simple or complex organisms, such as bacteria, 10 fungi (Timberlake and Marshall, 1989, *Science* 244:1313-7), invertebrates, plants (Gasser and Fraley, 1989, *Science* 244:1293), and mammals (Pursel *et al.*, 1989, *Science* 244:1281-8), which are rendered transgenic by the introduction of the heterologous cDNA.

15 For expression in mammalian cells, a cDNA sequence can be ligated to heterologous promoters, such as the simian virus SV40, promoter in the pSV2 vector (Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072-6), and introduced into cells, such as monkey COS-1 cells (Gluzman, 1981, *Cell* 23:175-82), to achieve transient or long-term expression. The stable integration of the chimeric gene construct may be maintained in mammalian cells by biochemical selection, such as neomycin (Southern and Berg, 1982, *J. Mol. Appl. Genet.* 1:327-41) and mycophoenolic acid (Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072-6).

20 The transfer of DNA into eukaryotic, such as human or other mammalian cells, is a conventional technique. The vectors are introduced into the recipient cells as pure DNA (transfection) by, for example, precipitation with calcium phosphate (Graham and vander Eb, 1973, *Virology* 52:466) strontium phosphate (Brash *et al.*, 1987, *Mol. Cell Biol.* 7:2013), electroporation (Neumann *et al.*, 1982, *EMBO J.* 1:841), lipofection (Felgner *et al.*, 1987, *Proc. Natl. Acad. Sci. USA* 84:7413), DEAE dextran (McCuthan *et al.*, 1968, *J. Natl. Cancer Inst.* 41:351), microinjection (Mueller *et al.*, 1978, *Cell* 15:579), protoplast fusion (Schafner, 1980, *Proc. Natl. Acad. Sci. USA* 77:2163-7), or pellet guns (Klein *et al.*, 1987, *Nature* 327:70). Alternatively, the cDNA can be introduced by infection with virus vectors, for example retroviruses (Bernstein *et al.*, 1985, *Gen. Engrg.* 7:235) such as adenoviruses (Ahmad *et al.*, 1986, *J. Virol.* 57:267) or Herpes (Spaete *et al.*, 25 1982, *Cell* 30:295).

EXAMPLE 14

Peptide Synthesis and Purification

30 The enzymes disclosed herein, such as alanine 2,3-aminomutase, CoA transferase, beta-alanyl-CoA ammonia lyase, 3-HP-CoA dehydratase, 4-aminobutyrate aminotransferase, beta-alanine-2-oxo-glutarate aminotransferase, 3-hydroxypropionate dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, glutamate dehydrogenase, 3-HP-CoA hydrolase, 3-hydroxyisobutryl-CoA hydrolase, poly hydroxyacid synthase, lipase, esterase, CoA hydrolase, alpha-ketopantoate

hydroxymethyltransferase, alpha-ketopantoate reductase, pantothenate synthase, pantothenate kinase, 4'-phosphopantethenoyl-1-cysteine synthetase, 4'-phosphopantethenoylcysteine decarboxylase, ATP:4'-phosphopantetheine adenyltransferase, dephospho-CoA kinase acetylating aldehyde:NAD(+) oxidoreductase, alcohol:NAD(+) oxidoreductase, aldehyde dehydrogenase (NAD(P)+) and alcohol

5 dehydrogenase (and variants, fusions, polymorphisms, fragments, and mutants thereof) can be chemically synthesized by any of a number of manual or automated methods of synthesis known in the art. For example, solid phase peptide synthesis (SPPS) is carried out on a 0.25 millimole (mmole) scale using an Applied Biosystems Model 431A Peptide Synthesizer and using 9-fluorenylmethyloxycarbonyl (Fmoc) amino-terminus protection, coupling with

10 dicyclohexylcarbodiimide/hydroxybenzotriazole or 2-(1H-benzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/hydroxybenzotriazole (HBTU/HOBT), and using *p*-hydroxymethylphenoxyethylpolystyrene (HMP) or Sasrin resin for carboxyl-terminus acids or Rink amide resin for carboxyl-terminus amides.

15 Fmoc-derivatized amino acids are prepared from the appropriate precursor amino acids by tritylation and triphenylmethanol in trifluoroacetic acid, followed by Fmoc derivitization as described by Atherton *et al.* (*Solid Phase Peptide Synthesis*, IRL Press: Oxford, 1989).

20 Sasrin resin-bound peptides are cleaved using a solution of 1% TFA in dichloromethane to yield the protected peptide. Where appropriate, protected peptide precursors are cyclized between the amino- and carboxyl-termini by reaction of the amino-terminal free amine and carboxyl-terminal free acid using diphenylphosphorylazide in nascent peptides wherein the amino acid sidechains are protected.

25 HMP or Rink amide resin-bound products are routinely cleaved and protected sidechain-containing cyclized peptides deprotected using a solution comprised of trifluoroacetic acid (TFA), optionally also comprising water, thioanisole, and ethanedithiol, in ratios of 100 : 5 : 5 : 2.5, for 0.5 - 3 hours at RT.

30 Crude peptides are purified by preparative high pressure liquid chromatography (HPLC), for example using a Waters Delta-Pak C18 column and gradient elution with 0.1% TFA in water modified with acetonitrile. After column elution, acetonitrile is evaporated from the eluted fractions, which are then lyophilized. The identity of each product so produced and purified may be confirmed by fast atom bombardment mass spectroscopy (FABMS) or electrospray mass spectroscopy (ESMS).

35 In view of the many possible embodiments to which the principles of our disclosure may be applied, it should be recognized that the illustrated embodiments are only particular examples of the disclosure and should not be taken as a limitation on the scope of the disclosure. Rather, the scope of the disclosure is in accord with the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.